

Nuclear Basic Fibroblast Growth Factor Regulation of Triple-Negative Breast Cancer

Dormancy/Recurrence

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Chemotherapy remains the only available treatment for triple-negative (TN) breast cancer. Although some TN breast cancers respond initially to neoadjuvant chemotherapy, the majority of patients die within three years of treatment due to recurrent tumor growth. Developing *ex vivo* models for TN breast cancer recurrence and defining responsible molecules will be crucial to developing effective combination therapies for TN breast cancer patients. We have developed an *in vitro* model of TN breast cancer dormancy/recurrence. Short-term exposure of tumor cells to chemotherapy at clinically relevant doses enriches for a dormant tumor cell population. Several days after removing chemotherapy, dormant tumor cells regain proliferative ability and establish colonies, resembling tumor recurrence. Tumor cells from “recurrent” colonies exhibit increased chemotherapy resistance, resembling therapy resistance of recurrent tumors in patients. Furthermore, we identify a novel signaling axis [nuclear bFGF/DNA-dependent protein kinase (DNA-PK)] supported by chemotherapy-enriched dormant TN breast cancer cells. This signaling axis drives accelerated DNA repair in chemo-residual TN breast cancer cells. Targeting this axis with either with a bFGF shRNA or DNA-PK small molecule inhibitor blocks recurrent colony formation. Using the Oncomine gene expression database, we found that bFGF expression in tumor samples from TN breast cancer patients predicts five-year tumor recurrence following

neoadjuvant chemotherapy treatment. Finally, we demonstrate that recurrent tumor cells exhibit increased invasiveness, reflecting the aggressive behavior of recurrent tumors in patients. Collectively, these studies identify a novel signaling axis in TN breast cancer that likely contributes to tumor recurrence and provide molecular targets for developing future therapeutics against TN breast cancer.

Dedication

This dissertation is dedicated to my Mom, Dad, and Joanna – Thank you for your unwavering support and endless love.

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List of Abbreviations

^3H	Tritium
AUG	codon for Methionine
BCA	bicinchoninic acid
BRCA1	breast cancer 1, early onset
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
cDNA	complementary DNA
cpm	counts per minute
CTC	circulating tumor cell
CUG	codon for Leucine
d	day
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PK _{cs}	DNA-dependent protein kinase catalytic subunit
Dox	doxorubicin
DSB	double strand repair

EDTA	Ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
EtOH	ethanol
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	hour
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HMW	high molecular weight
HR	homologous recombination
IC50	drug concentration for 50% inhibition
IgG	Immunoglobulin G
IRES	internal ribosome entry site
IV	intravenously
KCl	potassium chloride
kDa	kilo-dalton
LMW	low molecular weight

MET	mesenchymal-epithelial transition
MgCl ₂	magnesium chloride
min	minute
MMP	Matrix metalloproteinases
mRNA	messenger RNA
NHEJ	Non-homologous end joining
NIH	National Institutes of Health
NLS	nuclear localization signal
NP40	nonyl phenoxypolyethoxylethanol
NU7441	8-(4-Dibenzothieryl)-2-(4-morpholiny)-4H-1-benzopyran-4-one
P	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline (pH 7.4)
pH	power of hydrogen
PR	progesterone receptor
Rec	recurrent
RIPA	Radioimmunoprecipitation assay
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SD	standard deviation

SDS	Sodium dodecyl sulfate
siRNA	small inhibitory RNA
TGF β	transforming growth factor beta
TN	triple-negative
TNBC	triple-negative breast cancer
Tris	tris(hydroxymethyl)aminomethane
Tx	taxanes
ZEB1	Zinc finger E-box-binding homeobox 1

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1. Introduction

1.1 *Triple-negative (TN) breast cancer*

Breast cancer has been the most common type of cancer in women in the United States for many years. It is estimated that approximately 235,030 new cases of breast cancer and 40,430 breast cancer deaths will occur in US women in 2014[1]. The current treatment includes primary therapy, which is usually surgery, and adjuvant and neoadjuvant therapies, including chemotherapy, radiation, and targeted therapy. By interfering with specific molecules on tumor cells, targeted therapy, including hormonal agents and Trastuzumab, has greatly prolonged survival in the past few decades. However, targeted therapy is currently only available for breast cancers expressing estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2). Breast cancers that do not express these receptors cannot benefit from this therapeutic strategy. These tumors are often referred to as triple-negative breast cancers.

Triple-negative (TN) breast cancers are defined as tumors lacking expression of ER, PR, and HER2. They account for approximately 12-17% of all breast cancers in patients[2]. Although triple-negative breast cancers are very heterogeneous, as a group they are often associated with a worse prognosis, compared with receptor-positive breast cancers. TN breast cancer is associated with decreased 3-year survival rate, higher

risk for visceral metastasis, and shorter post-recurrence survival[3]. Patients with TN breast cancer have an increased likelihood of recurrence within 5 years of diagnosis[3, 4].

Besides the aggressive nature of TN breast cancer, the adverse outcome is largely due to the lack of targeted therapy. For patients with TN breast cancers, the only currently available systemic treatment is chemotherapy. Although there is no unanimous regimen for all TN breast cancer patients, the commonly used agents are doxorubicin, cyclophosphamide, and docetaxel, given short term for several cycles. Platinums, such as cisplatin and carboplatin, are currently being evaluated in clinical trials[2].

1.2 Tumor dormancy and recurrence

Despite receiving surgery and perioperative systemic treatment, patients with TN breast cancer often develop recurrent local or metastatic tumor with patency ranging from years to decades. This phenomenon can be explained by cancer dormancy: the chemo-residual tumor cells remain inactive and patients are asymptomatic[5]. The underlying mechanism of cancer dormancy is poorly understood. Compelling evidence show that a subpopulation of chemo-resistant cells can survive from chemotherapy and enter a non-proliferative quiescent state for many years before resuming growth, resulting in tumor recurrence (Fig. 1). This dormant state is alternatively referred to as cellular senescence[6-9]. Senescent cells can be identified by increased β -galactosidase, upregulation of p16, activation of p53, and active metabolism despite a G1 arrest[5].

Although senescence is traditionally considered irreversible, post-chemotherapy tumor cell senescence can be reversible, based on the evidence that tumor samples from breast cancer patients after chemotherapy stain β -gal and p16 positive[10]. Additionally, it has been reported that post-chemotherapy senescent cells can overcome cell cycle arrest at low frequency and resume proliferation *in vitro*[6, 9, 11]. Such senescent cell have characteristic morphologic features including enlarged and flattened shape, cytoplasmic granularity, and nuclear polyploidy[12].

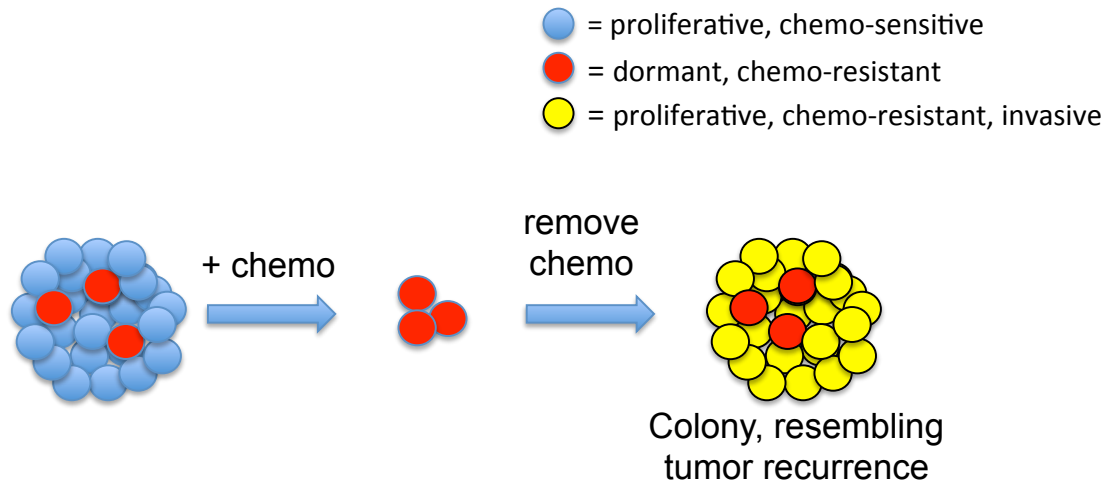


Figure 1: Model of tumor dormancy and recurrence. Individual tumor is heterogeneous. The original bulk of tumor contains chemo-sensitive cells (blue) and chemo-resistant cells (red). Chemotherapy eliminates chemo-sensitive tumor cells while leaving chemo-resistant cells intact. Residual chemo-resistant cells remain dormant for a period of time before resuming growth, resulting in tumor recurrence. Recurrent tumors are frequently more chemo-resistant, invasive, and fast growing.

1.3 Basic fibroblast growth factor (bFGF)

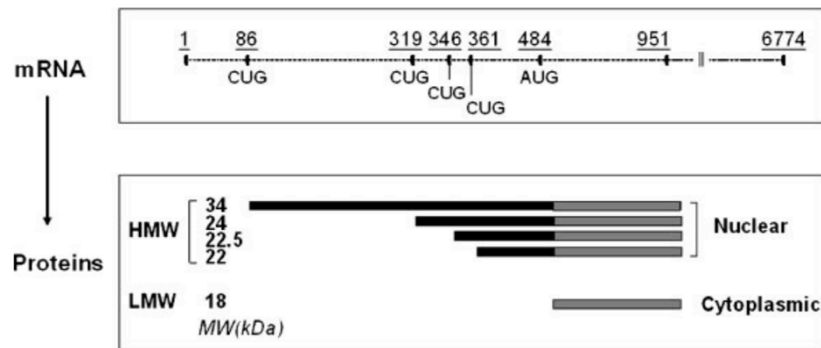
Basic fibroblast growth factor (bFGF, alternative name: FGF-2), a member of the fibroblast growth factor family, is ubiquitously conserved in eukaryotic organisms.

Although its primary function is stimulating fibroblast proliferation, bFGF is a multifunctional cytokine, regulating proliferation and differentiation of many different types of cells.

Human bFGF contains several isoforms: the 18 kDa low molecular weight (LMW) isoform originates from translation at the AUG initiation codon; the high molecular weight (HMW) isoforms, including 22, 22.5, and 24 kDa, originate from alternative translation at additional CUG initiation codons located on the same mRNA. An additional 34 kDa isoform is also observed but is often poorly translated. Unlike the LMW isoform, the HMW isoforms are produced through an internal ribosome entry site (IRES) by CAP-independent translation, which is often stimulated during cellular stress [13]. Besides the difference in translation mechanism, LMW and HMW isoforms have distinctive subcellular localization. The LMW isoform is predominantly localized in the cytosol, but is also released into extracellular space, for paracrine or autocrine (through receptor on cell surface) purpose. In contrast, the HMW isoforms are mainly localized in the nucleus due to nuclear localization sequences (NLS) (Fig. 2) [13]. However, recent studies revealed that the LMW isoform can also localize to the nucleus due to a

nonclassical NLS in the C-terminus[14, 15]. Conversely, a small number of HMW isoforms also present in the cytosol[13].

A



B

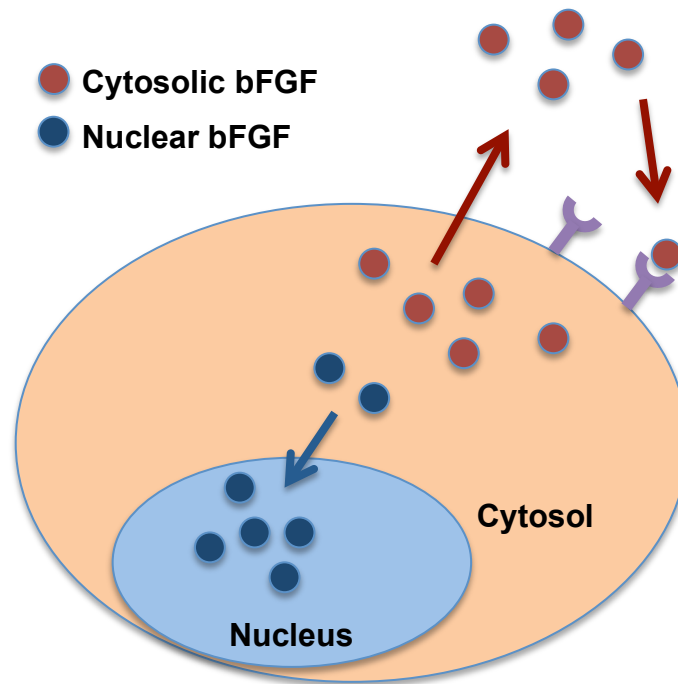


Figure 2: Cytosolic and nuclear isoforms of bFGF. A. Alternative translation results in LMW (18 kDa) bFGF and HMW (22, 22.5, 24, and 34 kDa) bFGF. Adapted from [13] **B.** Schematic illustration of bFGF subcellular localization. The LMW isoform (red) is predominantly localized in the cytosol, but is also secreted into extracellular space.

Through receptor(s) on the cell surface, LMW bFGF re-enters into cells to form an autocrine loop. In contrast, the HMW isoforms (blue) are produced in the cytoplasm but mainly localized in the nucleus. LMW, low molecular weight; HMW, high molecular weight.

Cytosolic and nuclear isoforms of bFGF have similar and different biological functions. Both of them can induce angiogenesis, increase cardiomyocyte proliferation, and stimulate neuronal regeneration, although through different signaling pathways[13]. However, only the 18 kDa cytosolic isoform induces cell migration, but not the nuclear isoforms[16]. In cell differentiation, undifferentiated human embryonic stem cells express both the cytosolic and nuclear isoforms of bFGF. However, after the induction of differentiation, only the cytosolic isoform is observed, indicating the role of nuclear isoforms in maintenance of pluripotency[17].

Furthermore, bFGF demonstrates important functions in cancer. Cytosolic (secreted) isoforms of basic fibroblast growth factor (bFGF) have been implicated in tumor resistance to anti-angiogenic therapy[18-23]. It has also been reported that cells overexpressing the 24 kDa isoform become radioprotected compared to the wild type or 18 kDa isoform-overexpressing cells[24]. Significantly, expression of the 24 kDa isoform in cancer cells strongly facilitates lung colonization and proliferation *in vivo*, and improves cancer cell survival under serum deprivation *in vitro*, indicating a unique function of nuclear bFGF on cancer cell survival and metastatic establishment[25]. Additionally, overexpression of the nuclear isoforms of bFGF correlates with a poor

prognosis in various human cancers[13]. To date, neither expression nor function of nuclear bFGF in breast cancer has been investigated.

1.4 DNA repair and DNA-dependent protein kinase (DNA-PK)

Repair of DNA double strand breaks (DSB) is critical for cell survival, especially for cancer cells under the pressure of radiation and DNA-damaging chemotherapy. Mammalian cells have two major pathways for DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ). HR is dependent on the presence of a sister chromatid and therefore is only available in the late S and G2 phases. In contrast, NHEJ, which occurs through out the cell cycle, joins the broken ends without the need of template, and is the predominant pathway for DSB repair[26, 27].

The key component of NHEJ is DNA-dependent protein kinase (DNA-PK). It contains the Ku70/80 heterodimer and the 460 kDa catalytic subunit (DNA-PK_{cs}). After DNA damage, Ku70/80 binds to the broken ends, recruits DNA-PK_{cs}, and stimulates its kinase activity. Autophosphorylation at threonine 2609 and serine 2056 sites on DNA-PK_{cs} is required for successful DSB repair. Therefore, DNA-PK activity is critical to confer radioresistance and chemoresistance in cancer cells[27-29]. Interestingly, overexpression of nuclear bFGF in HeLa cells upregulates the expression of DNA-PK_{cs} and confers protection from ionizing radiation[30]. Significantly, BRCA1, a tumor suppressor gene often dysregulated in TN breast cancer, repairs DNA double strand breaks mainly through the HR pathway[31, 32]. Due to frequent BRCA1 mutation, TN

breast cancer cells should have impaired DNA repair ability and therefore are vulnerable to DNA-damaging chemotherapy[2]. However, it is reasonable to postulate that cells with high DNA-PKcs expression may overcome the shortage of BRCA1 and thus become chemoresistant.

1.5 Epithelial-mesenchymal transition (EMT)

The epithelial-mesenchymal transition (EMT) was first characterized as a critical step during early embryogenesis[33]. Epithelial cells are closely connected to each other and basement membrane through tight junctions, gap junctions, desmosomes, and hemi-desmosomes. However, mesenchymal cells are loosely connected to each other and have increased migratory capability and invasiveness. The process of EMT is associated with downregulation of epithelial cell markers, such as E-cadherin and cytokeratin, and upregulation of mesenchymal cell markers, including N-cadherin, vimentin, and fibronectin. This process is regulated by EMT-inducing transcriptional factors (EMT-TFs), namely Snail, ZEB1, and Twist[34]. EMT is closely related to cancer metastasis. In cancers arising from epithelial tissues, primary tumor cells undergo EMT that empower them to invade into surrounding stroma tissue and intravasate to enter the blood circulation. These transformed mesenchymal-like tumor cells extravasate from blood vessels and seed in distal organs to form metastatic dissemination. These disseminated tumor cells may maintain dormancy for a period time until they repopulate to establish recurrent tumors[35].

1.6 Research objectives

The overall goal of this study is to understand the mechanisms underlying TN breast cancer dormancy/recurrence. We developed an *in vitro* model of tumor recurrence. Short-term chemotherapy treatment of TN breast cancer cells enriched for dormant, chemotherapy-resistant tumor cells. Upon removing chemotherapy, these dormant tumor cells resumed growth, resembling tumor recurrence. Based on the published importance of bFGF family in therapy resistance, we performed quantitative RT-PCR on chemo-enriched dormant cells and found upregulated bFGF transcription. We were surprised to see that nuclear but not cytosolic bFGF was upregulated in dormant tumor cells. Based on these initial findings, we sought to test the *hypothesis* that nuclear bFGF signaling drives TN breast cancer recurrence.

To establish an *in vitro* model for TN breast cancer dormancy/recurrence, we will:

1) investigate the ability of different chemotherapy classes (anthracyclines, taxanes) to enrich for a dormant TN tumor cell sub-population; and 2) determine whether these dormant cells establish recurrent colony formation upon chemotherapy removal.

To characterize the importance of bFGF signaling for TN breast cancer dormancy/recurrence, we will: 1) measure nuclear bFGF expression in chemotherapy-enriched dormant and recurrent TN tumor cells; 2) determine the function of bFGF in TN tumor dormancy/recurrence through knockdown and add back studies; and 3)

examine whether bFGF expression in tumor samples is associated with recurrence in TN breast cancer patients.

2. Experimental Procedures

2.1 Methods for establishment of in vitro model of TN breast cancer dormancy/recurrence

2.1.1 Cell culture

SUM159 triple-negative breast cancer cells were obtained from the Duke Cell Culture facility and maintained in Ham's F-12 medium containing 5% heat-inactivated FBS, 5 µg/ml insulin, and 1 µg/ml hydrocortisone. BT549 triple-negative breast cancer cells were obtained from the Duke Cell Culture Facility and maintained in RPMI 1640 containing 10% heat-inactivated FBS, 1 µg/ml insulin, 10 mM HEPES, 1 mM pyruvate, and 2.5 g/L glucose.

2.1.2 Generation of chemotherapy-enriched dormant tumor cells/“recurrent” colonies

SUM159 tumor cells were seeded in T225 cell culture flasks (2×10^6 cells/flask) and, after 2 d, treated with either 1 µg/ml doxorubicin (Sigma, St. Louis, MO) or 100 nM docetaxel (Sigma, St. Louis, MO). Drug was removed after 2 d, and cells were fed new medium every third day. The majority of cells (99.9%) were eliminated by day 8, after which only residual dormant cells (0.1%) were observed. BT549 tumor cells were seeded in T225 cell culture flasks (3×10^6 cells/flask) and, after 2 d, treated with 0.5 µg/ml doxorubicin. Drug was removed after 2 d, and cells were fed new medium every third day. The majority of cells (99.9%) were eliminated by day 8, after which only residual dormant cells (0.1%) were observed. SUM159 and BT549 dormant cells were harvested

on day 7 or 8 with trypsin-EDTA, and re-plated in 6-well plates. Medium was changed every 3-4 d. Recurrent colonies were stained with crystal violet and colonies containing > 50 cells were counted.

2.1.3 Time course- Cell death following acute chemotherapy treatment

SUM159 were incubated with doxorubicin (100 ng/ml) for 2 d, after which chemotherapy was removed, and new medium was added. Photographs were taken using an Olympus inverted microscope with a Canon EOS Rebel T4I. Final magnifications were 40X and 100X. Viable cell number was determined at 6 h, d1, d2, d3, and d7 using trypan blue exclusion.

2.1.4 Time course- Regrowth of chemo-residual tumor cells

Six days after chemotherapy removal, SUM159 cells were harvested with trypsin, and replated in 96 well plates (1000 cells/well). Tumor cell proliferation was assessed on a daily basis by measuring [³H]-thymidine uptake.

2.1.5 Western blots

Cells were harvested using trypsin-EDTA, washed with PBS, incubated in RIPA buffer on ice for 20 min, and then subjected to high speed centrifugation to obtain total cellular protein in the soluble fraction. For nuclear protein extraction, harvested cells were incubated in cytosolic lysis buffer [(10 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 0.5% NP40, and proteinase inhibitors)] on ice for 20 min and centrifuged.

Supernatants were collected as cytosolic protein lysates. The residual pellets were washed with cytosolic lysis buffer, and then incubated in nuclear lysis buffer [50 mM TRIS (pH 7.5), 1% SDS, and proteinase inhibitors] plus Benzonase (Sigma, St. Louis, MO) on ice for 20 min. The supernatants after centrifugation were collected as nuclear protein extracts. Protein concentrations were determined by BCA assay. Equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with the following primary antibodies, followed by the appropriate species IRDye-conjugated secondary antibody (Life Technologies, Carlsbad, CA): p21 (Cell Signaling, Beverly, MA), Actin (Sigma, St. Louis, MO), GAPDH (GenScript, Piscataway, NJ). Proteins were detected using an Odyssey infrared imaging system (LI-COR, Lincoln, NE).

2.1.6 Thymidine uptake assay

Thymidine Uptake: Cells were plated in 96-well plates (3×10^3 cells/well). After 4 h, cells were incubated with 0.5 μ Ci/well [3 H]-Thymidine (Perkin Elmer, Waltham, MA) for 16 h before harvesting onto glass-fiber filters. [3 H]-Thymidine incorporation was measured as counts per minute (CPM) using a Tri-Carb 2100TR time-resolved liquid scintillation counter (Perkin Elmer, Waltham, MA).

2.1.7 Alamar blue cell viability assay

Cells were plated in 96-well black, clear bottom plates (2×10^3 cells/well) in 100 μ l complete medium. After 4 h, 10 μ l/well Alamar Blue (Life Technologies, Carlsbad, CA)

reagent was added. After 2 h, fluorescence was measured using a Cytation3 plate reader (BioTek, Winooski, VT)

2.1.8 PKH26 labeling study

SUM159 cells were labeled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. The labeled SUM159 cells were treated with doxorubicin (1 $\mu\text{g/ml}$) to generate chemotherapy enriched dormant cells, as described above. Labeled cells were detected using the Guava EasyCyte Plus flow cytometer (Millipore, Billerica, MA).

2.2 Methods for mechanistic studies of TN breast cancer dormancy/recurrence

2.2.1 Cell culture

SUM159 triple-negative breast cancer cells were obtained from the Duke Cell Culture facility and maintained in Ham's F-12 medium containing 5% heat-inactivated FBS, 5 $\mu\text{g/ml}$ insulin, and 1 $\mu\text{g/ml}$ hydrocortisone. BT549 triple-negative breast cancer cells were obtained from the Duke Cell Culture Facility and maintained in RPMI 1640 containing 10% heat-inactivated FBS, 1 $\mu\text{g/ml}$ insulin, 10 mM HEPES, 1 mM pyruvate, and 2.5 g/L glucose.

2.2.2 Generation of chemotherapy-enriched dormant tumor cells/ "recurrent" colonies

SUM159 tumor cells were seeded in T225 cell culture flasks (2×10^6 cells/flask) and, after 2 d, treated with either 1 $\mu\text{g/ml}$ doxorubicin (Sigma, St. Louis, MO) or 100 nM

docetaxel (Sigma, St. Louis, MO). Drug was removed after 2 d, and cells were fed new medium every third day. The majority of cells (99.9%) were eliminated by day 8, after which only residual dormant cells (0.1%) were observed. BT549 tumor cells were seeded in T225 cell culture flasks (3×10^6 cells/flask) and, after 2 d, treated with 0.5 $\mu\text{g/ml}$ doxorubicin. Drug was removed after 2 d, and cells were fed new medium every third day. The majority of cells (99.9%) were eliminated by day 8, after which only residual dormant cells (0.1%) were observed. SUM159 and BT549 dormant cells were harvested on day 7 or 8 with trypsin-EDTA, and re-plated in 6-well plates. Medium was changed every 3-4 d. Recurrent colonies were stained with crystal violet and colonies containing > 50 cells were counted.

2.2.3 Western blots

Cells were harvested using trypsin-EDTA, washed with PBS, incubated in RIPA buffer on ice for 20 min, and then subjected to high speed centrifugation to obtain total cellular protein in the soluble fraction. For nuclear protein extraction, harvested cells were incubated in cytosolic lysis buffer [10 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl_2 , 0.5% NP40, and proteinase inhibitors] on ice for 20 min and centrifuged. Supernatants were collected as cytosolic protein lysates. The residual pellets were washed with cytosolic lysis buffer, and then incubated in nuclear lysis buffer [50 mM TRIS (pH 7.5), 1% SDS, and proteinase inhibitors] plus Benzonase (Sigma, St. Louis, MO) on ice for 20 min. The supernatants after centrifugation were collected as nuclear protein

extracts. Protein concentrations were determined by BCA assay. Equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with the following primary antibodies, followed by the appropriate species IRDye-conjugated secondary antibody (Life Technologies, Carlsbad, CA): bFGF (BD Biosciences, Franklin Lakes, NJ), Lamin-A (Sigma, St. Louis, MO), DNA-PK ϵ s (Cell Signaling, Beverly, MA), phospho-Ser 2056-DNA-PK ϵ s (Cell Signaling, Beverly, MA) GAPDH (GenScript, Piscataway, NJ). Proteins were detected using an Odyssey infrared imaging system (LI-COR, Lincoln, NE).

2.2.4 Immunofluorescence

Cells were grown on glass coverslips, washed with Hanks' balanced salt solution (HBSS), and fixed for 30 min at room temperature in 1% fresh formaldehyde in PBS. After washing for 5 min in PBS, the coverslips were incubated in 5% bovine serum albumin (BSA) in PBS for 90 min at room temperature. Excess BSA was drained from the coverslips, and cells were incubated with the primary bFGF antibody (BD Biosciences, Franklin Lakes, NJ) in PBS containing 0.5% BSA for overnight at 4 °C. The cells were then rinsed three times in PBS containing 0.1% Tween and incubated with secondary antibody (1:400 dilution of an Alexa Fluor 568-conjugated donkey anti-mouse IgG) for 90 min at 4 °C in the dark. The cells were washed three times with PBS, incubated with a 1:1000 dilution of Hoechst (Life Technologies, Carlsbad, CA) for 10 min at room temperature in the dark, and washed three times with PBS. The coverslips were dried

for 2 h at room temperature, mounted, and cured overnight at 4 °C. Pictures were taken using a fluorescence microscope and analyzed by Gen5 image analysis software (BioTek, Winooski, VT).

2.2.5 Thymidine uptake assay

Cells were plated in 96-well plates (3×10^3 cells/well). After 4 h, cells were incubated with 0.5 μ Ci/well [3 H]-Thymidine (Perkin Elmer, Waltham, MA) for 16 h before harvesting onto glass-fiber filters. [3 H]-Thymidine incorporation was measured as counts per minute (CPM) using a Tri-Carb 2100TR time-resolved liquid scintillation counter (Perkin Elmer, Waltham, MA).

2.2.6 Alamar blue cell viability assay

Cells were plated in 96-well black, clear bottom plates (2×10^3 cells/well) in 100 μ l complete medium. After 4 h, 10 μ l/well Alamar Blue (Life Technologies, Carlsbad, CA) reagent was added. After 2 h, fluorescence was measured using a Cytation3 plate reader (BioTek, Winooski, VT)

2.2.7 shRNA and add back transfections

Cells were grown to 50% confluence in a 10 cm dish. The transfection mixtures contained: 1) 2 μ g bFGF or control shRNA plasmid (Sigma, St. Louis, MO) with 250 μ l Optimem (Life Technologies, Carlsbad, CA) and 2) 3.8 μ l of Lipofectamine 2000 (Life Technologies, Carlsbad, CA) with 250 μ l Optimem. These mixtures were incubated separately at room temperature for 5 min, combined, and incubated for 30 min at room

temperature. Cells were washed 2X with HBSS (Life Technologies, Carlsbad, CA). Optimem (5.5 ml) was then added to the RNA/Lipofectamine mixture, and this mix was added to the cells, which were incubated overnight at 37°C. This medium was removed the next day and replaced with medium containing puromycin (5 µg/mL, SUM159; 2 µg/mL, BT549). Cells were expanded in puromycin and tested for bFGF knockdown by western blotting. For bFGF add backs, plasmids(19) expressing 18 kDa rat bFGF, 23 kDa rat bFGF, or an empty control were transfected into SUM159 or BT549 cells stably expressing a bFGF shRNA . The transfection protocol was performed as above, except that the cells were selected in puromycin (as above) and G418 (Life Technologies, Carlsbad, CA) at 400 µg/ml. Expression of addback constructs was assessed by western blotting extracts with bFGF antibody.

2.2.8 Single cell gel electrophoresis assay (Comet assay)

Cells were challenged with doxorubicin (SUM159: 1 µg/ml, 3h; BT549: 0.5 µg/ml, 4h). Fresh medium was added after chemotherapy removal. Cells were harvested at sequential time points after chemotherapy, mixed with low-melting-point agarose, and spread on CometSlides using a Trevigen CometAssay® Kit (Gaithersburg, MD). After incubation with lysis solution and neutral solution, slides were subjected to electrophoresis at 19 V for 50 min under neutral conditions. Slides were incubated with DNA precipitation solution (1 M NH₄AC, 95% EtOH) for 30 min, followed by 70% ethanol for 30 min. Slides were then stained with a 1:500 dilution of Hoechst (Life

Technologies, Carlsbad, CA) for 15 min and washed with PBS. Samples were examined using a fluorescence microscope, and the presence of comet tails was quantified using Gen5 image analysis software (BioTek, Winooski, VT). For each time point, cells from three fields were analyzed. Each field contained at least 50 cells.

2.2.9 Real-time quantitative RT-PCR

Total RNA from SUM159 cells was extracted using PrepEase® RNA Spin Kit (USB, Cleveland, OH) and treated with RNase-free DNase to remove residual genomic DNA. Single-stranded cDNAs were synthesized using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). Human FGF2 and human DNA-PKcs primers were purchased from realtimeprimers.com (Elkins Park, PA). Real-time PCR on the Mx3005P® QPCR System (Stratagene, La Jolla, CA) was performed in the presence of 12.5 µl VeriQuest™ Fast SYBR Green qPCR Master mix(2x) (USB, Cleveland, OH), 2 µl cDNA, and H₂O added to a final volume of 25 µl. The mixtures were denatured for 5 min at 95°C, followed by 40 cycles of 3 s at 95°C, and 30 s at an annealing temperature at 60°C. PCR products were monitored in real time by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye. Significance was analyzed using the software package MxPro™ QPCR Software (Stratagene, La Jolla, CA).

2.2.10 Selective DNA-PK inhibitor (NU7441) studies

Cells were seeded in T225 cell culture flasks (2 x 10⁶ cells/flask) and, after 2 d, treated with 1 µg/ml doxorubicin (Sigma, St. Louis, MO) plus DMSO or NU7441 (1 µM

or 5 μ M, R&D Systems, Minneapolis, MN). Drug was removed after 2 d, and cells were fed with new medium every third day. Dormant cells were harvested on day 7 with trypsin-EDTA, and re-plated in 6-well plates. Medium was changed every 3-4 d. Recurrent colonies were stained with crystal violet and colonies containing > 50 cells were counted.

2.2.11 Microarray data analysis

The normalized expression values for bFGF from a breast cancer gene profile study[36] were obtained from the Oncomine cancer gene expression database. The subset data of triple-negative breast cancer was pulled. The five year tumor recurrence status of patients was compared to bFGF expression from pre-treatment biopsy samples. Results were presented using the Box-Whistler plot and p values were calculated by the Mann-Whitney U test.

2.3 Methods for characterization of recurrent TN breast cancer cells

2.3.1 Cell culture

SUM159 triple-negative breast cancer cells were obtained from the Duke Cell Culture facility and maintained in Ham's F-12 medium containing 5% heat-inactivated FBS, 5 μ g/ml insulin, and 1 μ g/ml hydrocortisone.

2.3.2 Generation of chemotherapy-enriched dormant tumor cells/“recurrent” colonies

SUM159 tumor cells were seeded in T225 cell culture flasks (2×10^6 cells/flask) and, after 2 d, treated with either 1 $\mu\text{g/ml}$ doxorubicin (Sigma, St. Louis, MO) or 100 nM docetaxel (Sigma, St. Louis, MO). Drug was removed after 2 d, and cells were fed new medium every third day. The majority of cells (99.9%) were eliminated by day 8, after which only residual dormant cells (0.1%) were observed. SUM159 dormant cells were harvested on day 7 or 8 with trypsin-EDTA, and re-plated in 6-well plates. Medium was changed every 3-4 d. Recurrent colonies were stained with crystal violet and colonies containing > 50 cells were counted.

2.3.3 Western blots

Cells were harvested using trypsin-EDTA, washed with PBS, incubated in RIPA buffer on ice for 20 min, and then subjected to high speed centrifugation to obtain total cellular protein in the soluble fraction. For nuclear protein extraction, harvested cells were incubated in cytosolic lysis buffer [10 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl_2 , 0.5% NP40, and proteinase inhibitors] on ice for 20 min and centrifuged. Supernatants were collected as cytosolic protein lysates. The residual pellets were washed with cytosolic lysis buffer, and then incubated in nuclear lysis buffer [50 mM TRIS (pH 7.5), 1% SDS, and proteinase inhibitors] plus Benzonase (Sigma, St. Louis, MO) on ice for 20 min. The supernatants after centrifugation were collected as nuclear protein extracts. Protein concentrations were determined by BCA assay. Equivalent amounts of

protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with the following primary antibodies, followed by the appropriate species IRDye-conjugated secondary antibody (Life Technologies, Carlsbad, CA): N-cadherin (Thermo Fisher, Waltham, MA), Pan-cytokeratin (Cell Signaling, Beverly, MA), MMP-2 (Millipore, Billerica, MA), c-Jun (Cell Signaling, Beverly, MA), phospho-Ser 63-c-Jun (Cell Signaling, Beverly, MA), bFGF (BD Biosciences, Franklin Lakes, NJ), Lamin-A (Sigma, St. Louis, MO), DNA-PKcs (Cell Signaling, Beverly, MA), phospho-Ser 2056-DNA-PKcs (Cell Signaling, Beverly, MA), Histone H2A.X (Cell Signaling, Beverly, MA), GAPDH (GenScript, Piscataway, NJ). Proteins were detected using an Odyssey infrared imaging system (LI-COR, Lincoln, NE).

2.3.4 Thymidine uptake assay

Cells were plated in 96-well plates (2×10^3 cells/well). After overnight incubation, cells were treated with doxorubicin (Sigma, St. Louis, MO) at different concentrations (0-100 ng/ml) or docetaxel (Sigma, St. Louis, MO) at different concentrations (0-10 nM) for 48 h. Cells were incubated with 0.5 μ Ci/well [3 H]-Thymidine (Perkin Elmer, Waltham, MA) for 16 h before harvesting onto glass-fiber filters. [3 H]-Thymidine incorporation was measured as counts per minute (CPM) using a Tri-Carb 2100TR time-resolved liquid scintillation counter (Perkin Elmer, Waltham, MA).

2.3.5 Wound-healing migration assay

Tumor cells from recurrent colonies, as well as parental tumor cells, were plated on a 24-well plate and grew to reach confluence. Cells in the middle of each well were mechanically scratched using a 20 μ l pipette tip. Cell debris were removed by PBS wash. Fresh medium was added. Images were taken on 0 h and 8 h using an Olympus inverted microscope with a Canon EOS Rebel T4I. At least three random locations were examined and representative pictures were shown.

2.3.6 Transwell invasion assay

Tumor cells from recurrent colonies, as well as parental tumor cells, were serum-starved for 24 h. Equal amounts of cells were added to the upper chambers of a matrigel-coated plate (Corning, Tewksbury, MA). Completed medium was added to the bottom chambers. After 12 h, cells that migrated into the bottom chambers were fixed and stained with crystal violet. The number of migrated cells was counted from three fields using an Olympus inverted microscope with a Canon EOS Rebel T4I.

3. Development of an *in vitro* Model of TN Breast Cancer Dormancy/Recurrence

3.1 Introduction

Despite the apparent efficacy of chemotherapy in “shrinking” primary tumors, chemotherapy-resistant tumor cells are thought to contribute to future tumor recurrence, the leading cause of patient mortality. The identification of proteins that confer chemotherapy resistance has historically relied on studies of signaling pathways supported by tumor cells subjected to long-term drug selection. These long-term selection models select for mutations/epigenetic modifications that result in acquired expression/activity of proteins involved in therapy resistance.

Other models propose that tumors are heterogeneous, consisting of therapy-sensitive and therapy-resistant tumor cell subpopulations. According to these models, following chemotherapy treatment, chemo-resistant tumor cells exist in a dormant (sleeping) state for many years before resuming growth, resulting in tumor recurrence. Methods are needed to enrich for dormant tumor cells, allowing for studies of their unique signaling properties. Such studies will be critical to defining logical therapeutic targets for preventing tumor recurrence.

Using short term chemotherapy treatment to enrich for drug-resistant tumor cells, we have developed an *in vitro* model of tumor recurrence. In this model, short-term exposure of TN breast tumor cells to clinical-relevant chemotherapy classes/doses enriches for a population of slow-cycling (dormant) tumor cells. Chemotherapy-

enriched dormant tumor cells resume proliferation after chemotherapy withdrawal, forming colonies resembling a tumor recurrence. Contrasting with evolution models of therapy resistance, the existence of drug-resistant tumor cell subpopulations in the original tumor suggests that we can effectively eliminate tumor recurrence by implementing combination therapies (chemotherapy targeting proliferative cells + therapy targeting drug-resistant dormant cells).

3.2 Results

3.2.1 Generation of dormant tumor cells and “recurrent” colonies by doxorubicin

Several studies indicate that drug-resistant, slow-cycling tumor cells are represented at low frequency in human tumors, and are therapy resistant [37, 38]. The contribution of these cells to tumor recurrence following chemotherapy treatment is not known. We investigated the hypothesis that short-term exposure of tumor cells to chemotherapy enriches for a slow-cycling, chemo-resistant tumor cell sub-population that can, over time, resume growth, thus resembling tumor recurrence. To test this hypothesis, we exposed human TN breast cancer cells to acute chemotherapy treatment. SUM159 and BT549 TN breast tumor cells were exposed to doxorubicin at clinical-relevant concentration (1 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$, respectively)[39]. Doxorubicin was removed on d2, and fresh culture medium was added. After 7 or 8 days (SUM159), the majority of tumor cells were dead. However, we noted that a small number of residual tumor cells remained. These residual tumor cells appeared to be non-proliferative, as

indicated by the fact that their numbers did not increase for several days (data not shown). Approximately 12 d after chemotherapy removal, these residual tumor cells resumed proliferation and eventually formed colonies, resembling a tumor recurrence (Fig. 3).

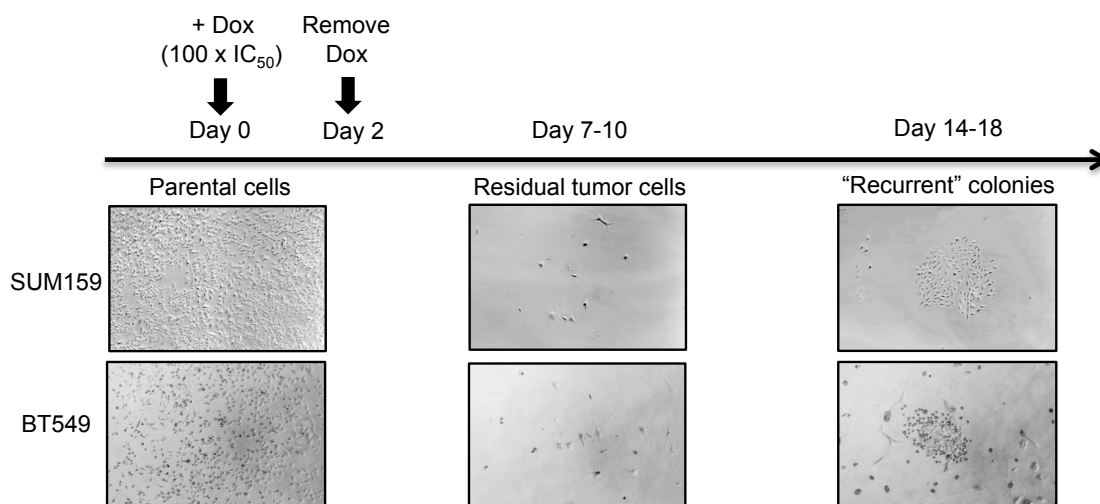


Figure 3: Generation of dormant tumor cells and "recurrent" colonies by doxorubicin[40]. SUM159 and BT549 TN breast tumor cells were treated with doxorubicin (1 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, respectively) for 2 d, after which chemotherapy was removed and fresh medium was added. Between 7 and 10 d, a small number of dormant cells (0.1% of the original population) remained, and exhibited significantly reduced proliferation compared to parental (untreated) cells. Approximately 2 weeks after chemotherapy withdrawal, the dormant cells resumed proliferation and established "recurrent" colonies. Pictures of parental (untreated), dormant, and recurrent cells were taken on d 0, 7, 14 (SUM159 cell) or d 0, 10, 18 (BT549 cell), respectively. Magnification 20X.

3.2.2 Generation of dormant tumor cells and “recurrent” colonies by docetaxel

To determine whether chemo-resistant tumor cell sub-population can be enriched by microtubule-modifying agents, we exposed SUM159 TN breast tumor cells to docetaxel (100 nM). Docetaxel was removed on d2 for SUM159 cells, and fresh culture medium was added. After 8 days, the majority of tumor cells were dead. However, we noted that a small number of residual tumor cells remained. These residual tumor cells appeared to be non-proliferative, as indicated by the fact that their numbers did not increase for several days (data not shown). Approximately 10 d after chemotherapy removal, these residual tumor cells resumed proliferation and eventually formed colonies, resembling a tumor recurrence (Fig. 4).

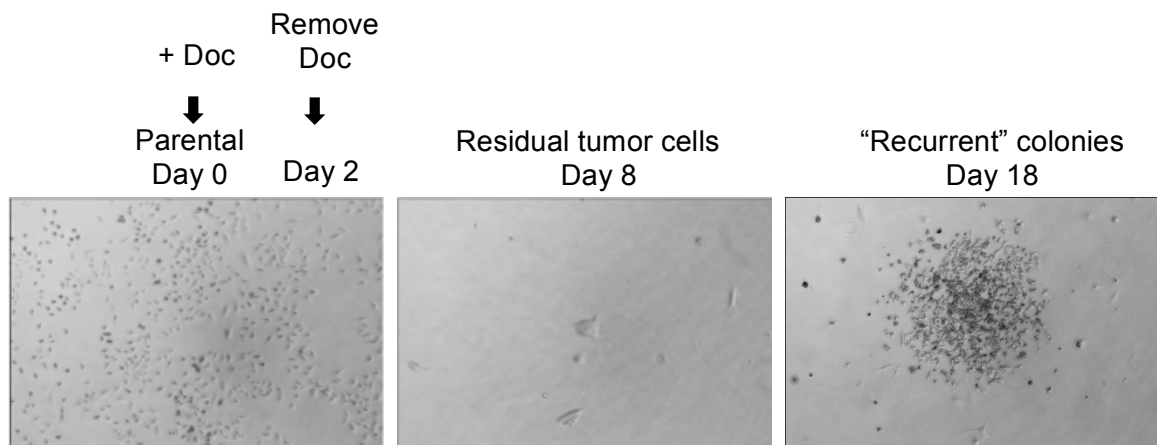


Figure 4: Generation of dormant tumor cells and “recurrent” colonies by docetaxel[41]. SUM159 TN breast tumor cells were treated with docetaxel (100 nM) for 2 d, after which chemotherapy was removed and fresh medium was added. Between 7 and 10 d, a small number of dormant cells (0.1% of the original population) remained, and exhibited significantly reduced proliferation compared to parental (untreated) cells.

Approximately 2 weeks after chemotherapy withdrawal, the dormant cells resumed proliferation and established “recurrent” colonies. Pictures of parental (untreated), dormant, and recurrent cells were taken on d 0, 8, 18, respectively. Magnification 20X.

3.2.3 Morphology of dormant tumor cells

Previous studies suggest that short-term chemotherapy can induce reversible cellular senescence that is associated with chemoresistance in several types of cancer, including breast cancer[6, 11, 12]. Correspondingly, the chemotherapy-enriched dormant cells in our model also have distinctive morphologic features--flattened and enlarged cytoplasm (>5 times larger than untreated parental cells), similar to senescent-like morphology (Fig. 5).

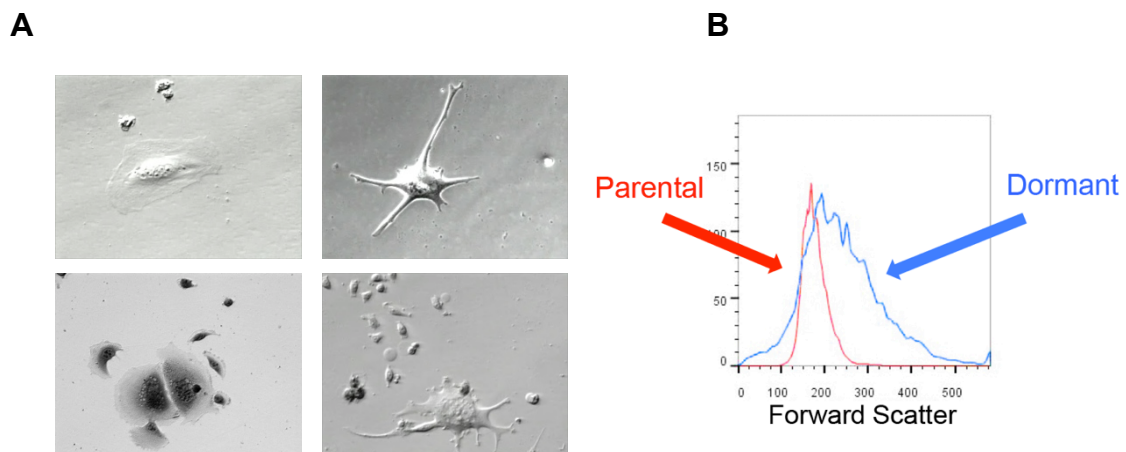


Figure 5: Morphology of dormant tumor cells. **A.** Pictures of dormant cells on showing distinctive morphologic features: enlarged cytoplasm, “fried egg” appearance, or stellar protrusions. Note the two lower pictures also contain recurrent cells that are much smaller in size. Magnification 100X. **B.** Flow cytometry shows increased cell size (as indicated by forward scatter) in chemotherapy-enriched dormant tumor cells compared to untreated parental cells.

3.2.4 Pre-existence of dormant tumor cells in parental tumor cell lines

Previous studies indicate that tumors are heterogeneous, consisting of distinct tumor cell subpopulations[42, 43]. To determine if the dormant cells generated in our model after short-term chemotherapy exposure represent a subpopulation of cells in the original tumor cell line, we performed a label retention study using the label retaining dye PKH26. Unlike parental tumor cells, which lose this dye upon cell division, dormant tumor cells surviving chemotherapy treatment retained this dye (Fig. 6). This finding indicates that dormant tumor cells generated in our chemotherapy model are non-proliferative or slowly proliferating tumor cells represented in the parental tumor cell line.

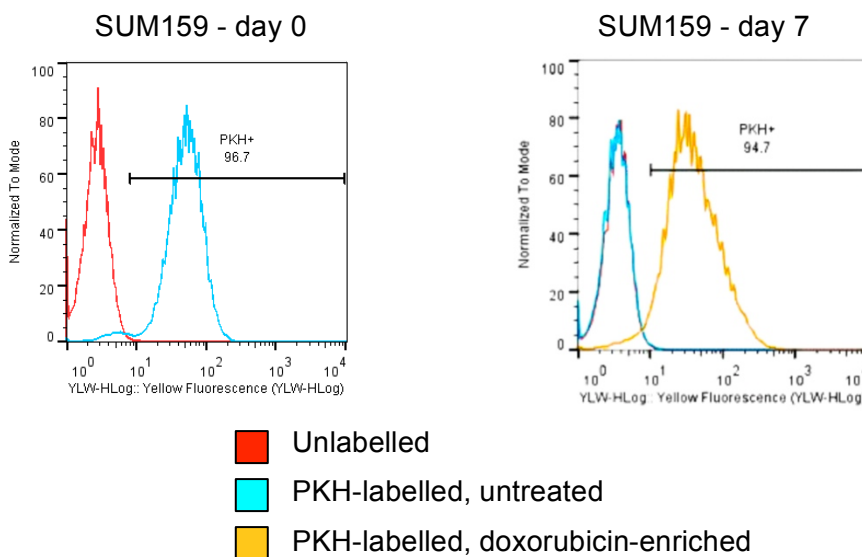


Figure 6: Dormant tumor cells are represented in parental tumor cell lines and enriched by chemotherapy treatment[41]. SUM159 cells were stained with the label-retaining dye PKH26. PKH26-labelled cells were either left untreated (blue line) or

incubated for 2 d with doxorubicin (1 $\mu\text{g/ml}$; yellow line). The percentage of label-retaining cells was determined on d8 after treatment. Note that at the time of harvest, untreated cells (proliferative) had lost the dye, whereas pre-existing dormant cells enriched by chemotherapy had retained the dye.

3.2.5 Dormant tumor cells exhibit reduced proliferation but metabolically active state

In its most simple form, tumor dormancy has been defined as a condition in which residual cancer cells stop dividing[44]. It is thought that these cells remain dormant for a prolonged period before receiving signals (intrinsic or extrinsic) that cause them to resume growth and establish recurrent tumors. Fitting this definition of dormancy, both SUM159 and BT549 cells surviving short term chemotherapy in our model represented a sub-population of cells that did not take up appreciable thymidine, but were metabolically active, as indicated using an alamar blue assay (Fig. 7A and B). Notably, chemo-residual BT549 cells exhibited increased alamar blue positivity compared to parental BT549 cells, suggesting that these enriched cells may have elevated metabolism. Chemo-residual tumor cells also expressed increased levels of p21 (Fig. 7C), a cell cycle arrest protein.

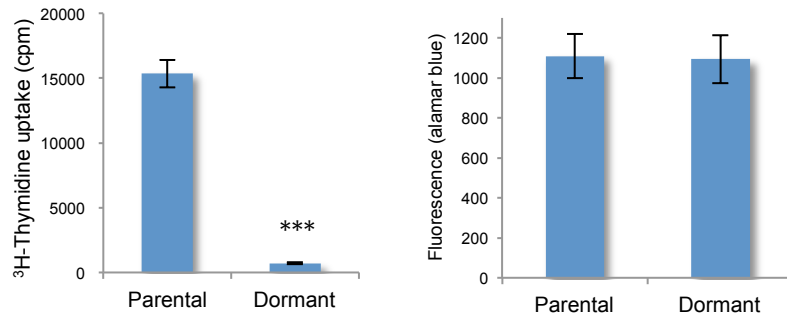
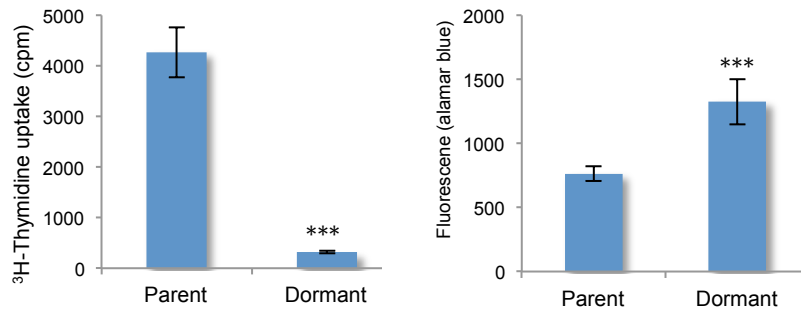
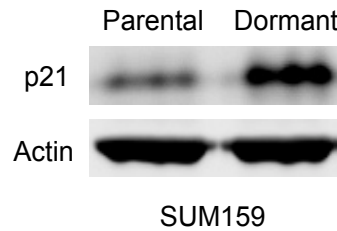
A**B****C**

Figure 7: Dormant tumor cells maintain in a non-proliferative but metabolically active state[40, 41]. A and B. SUM159 (A) and BT549 (B) cells were exposed to acute doxorubicin treatment (as described in Fig. 1). Residual tumor cells surviving short-term chemotherapy treatment were harvested on d8, and seeded at 2000 cells/well in triplicate wells of a 96 well plate. Proliferation was determined by [³H]-thymidine incorporation (+/-SD). Cell viability was assessed by alamar blue (fluorescence +/- SD). n=6, error bars represent S.D., ***p<0.001, two-tailed student's t-test. **C.** Total cellular protein was extracted from parental and dormant SUM159 cells, and equivalent amounts were immunoblotted with p21 antibody, followed by IrDye-conjugated secondary antibody. Protein loading was assessed using Actin antibody. Similar results were obtained in three independent trials.

3.2.6 Dormant tumor cells resume growth and establish recurrent colonies

We next sought to determine the time after chemotherapy removal that dormant tumor cells resumed growth after chemotherapy removal. The number of viable breast tumor cells decreased for five days after chemotherapy removal, as demonstrated in Fig. 8A and B. However, residual tumor cells did not resume proliferation until approximately 10 days after chemotherapy removal, as assessed by [³H]-thymidine uptake (Fig. 8C). Similar kinetics of growth were observed using the BT549 cell line (data not shown).

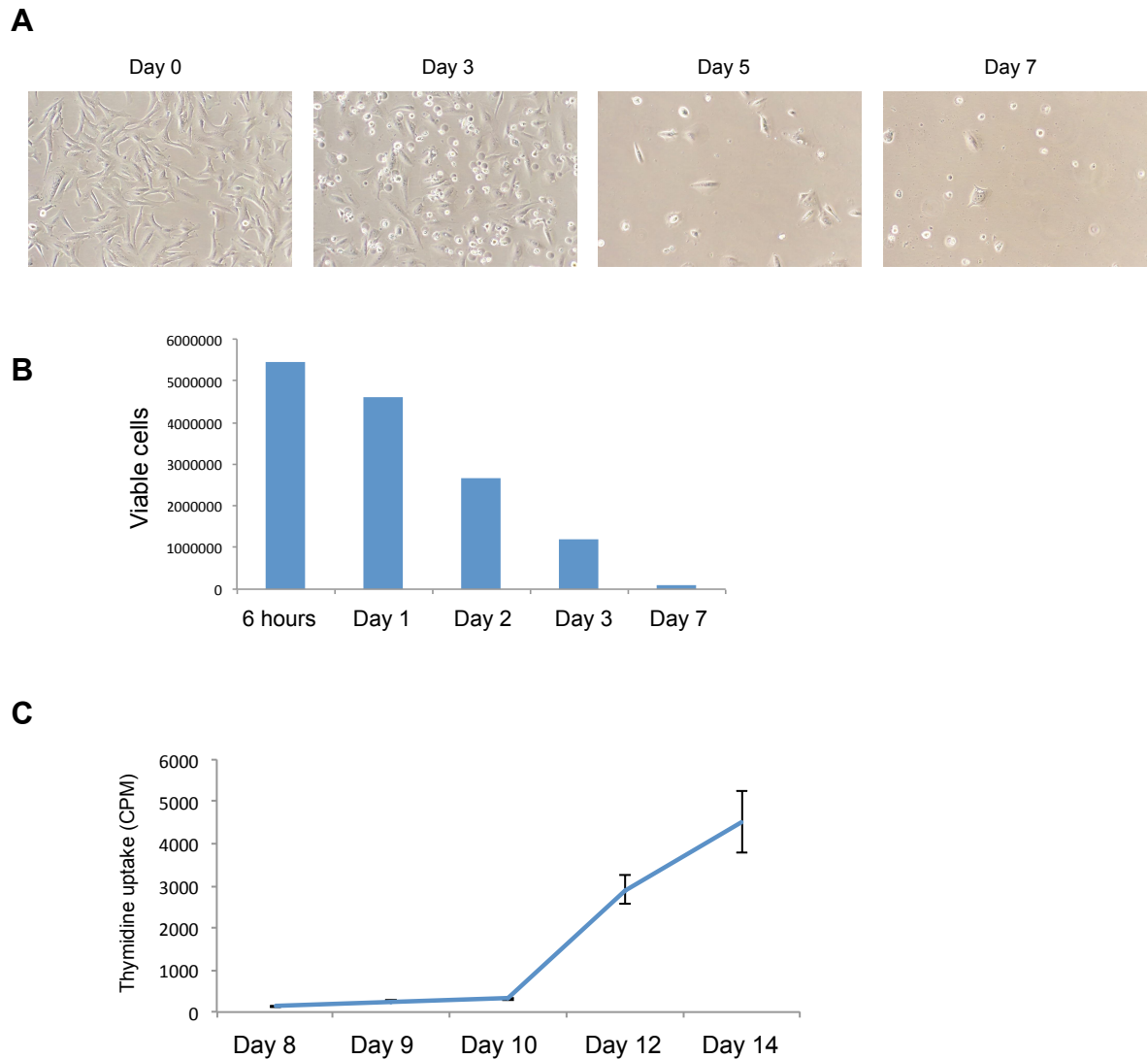


Figure 8: Kinetics of recurrent colony growth[41]. SUM159 tumor cells were incubated with doxorubicin (2d) as indicated in Fig.1. **A and B.** Kinetics of cell die-off were assessed by imaging representative fields (A) as well as by counting viable cells using trypan blue (B) at the indicated times. **C.** Proliferative status of residual tumor cells was measured over time by performing [^3H]-thymidine incorporation assays on cells harvested at the indicated times (2000 cells/well).

3.3 Discussion

Our results demonstrate that dormant, chemo-resistant tumor cells can be enriched from breast cancer cell lines by short-term chemotherapy treatment. DNA-damaging (doxorubicin) and microtubule-modifying (docetaxel) chemotherapies, representing standard treatment regimens for TN breast cancer patients, enriched for these dormant cells at clinically relevant doses[45, 46], indicating broad relevance to patient treatment (Fig. 1A and B).

Traditional models of tumor recurrence focus on the modifications occurring in tumor cells upon long-term drug selection. These long-term selection models select for mutations/epigenetic modifications that result in acquired expression/activity of proteins involved in therapy resistance[38, 47]. In contrast, our model enriches for the “culprit” dormant tumor cells by short term chemotherapy.

Our *in vitro* model of tumor dormancy/recurrence is important because it enriches for a dormant tumor cell population that is normally under-represented in the parental tumor cell line. This model provides us a platform to facilitate studies on identifying novel signaling pathways that drive tumor dormancy/recurrence. These studies have the potential to identify: 1) logical therapeutic targets on chemo-resistant, dormant tumor cell populations, and 2) biomarkers that predict recurrence-free survival.

4. bFGF Regulation of TN Breast Cancer Dormancy/Recurrence

4.1 Introduction

Although tumor recurrence is frequently observed after chemotherapy treatment of aggressive breast cancers, little is known about the signaling pathways that contribute to TN breast cancer recurrence.

The basic fibroblast growth factor family (bFGF; alternative name: FGF-2) consists of both cytosolic (secreted) and nuclear isoforms. Expression of these bFGF isoforms is regulated at the level of translation. Specifically, cytosolic isoforms (low molecular weight, 18 kDa) are regulated by CAP-dependent translation, whereas nuclear isoforms (high molecular weight; 22, 22.5, and 24 kDa) are regulated by CAP-independent translation[48]. These isoforms differ in molecular weight because they utilize different translation initiation sites.

Cytosolic (secreted) isoforms of basic fibroblast growth factor (bFGF) have been implicated in tumor resistance to anti-angiogenic therapy[18-23]. However, functions for nuclear bFGF in cancer cells remain poorly understood. In over-expression models, nuclear bFGF has been reported to regulate cell cycle[49-51], cell survival[25], radioresistance[30], and tumor metastasis[25, 52]. Moreover, nuclear bFGF expression in astrocytic tumors is associated with a poor patient prognosis[53]. To date, nuclear bFGF expression/function in breast cancer has not been investigated.

DNA repair pathways are frequently de-regulated in breast cancer. While BRCA proteins are responsible for homologous repair, DNA-dependent protein kinase (DNA-PK) repairs double-stranded DNA breaks by non-homologous end joining. DNA-PK consists of a catalytic subunit (DNA-PKcs) and a regulatory subunit (Ku70/80 heterodimer), which recruit DNA-PKcs to DNA. The status of the cell cycle determines whether DNA-PK or BRCA repairs DNA, with DNA-PK being responsible in growth arrested cells[54].

Previous studies using bFGF overexpression models suggest that nuclear bFGF drives DNA-PKcs transcription[30], but an ability of endogenous bFGF to regulate DNA-PKcs expression/DNA repair in tumor cells has not been reported. In the current work, we show that chemo-residual dormant TN tumor cells are dependent on a nuclear bFGF/DNA-PKcs signaling axis for their survival and resumed proliferation following chemotherapy treatment. Our work identifies a novel signaling axis that likely contributes to TN breast cancer recurrence.

4.2 Results

4.2.1 Chemotherapy-enriched dormant cells have increased bFGF mRNA level

To determine whether bFGF expression is associated with tumor dormancy, we performed quantitative RT-PCR analysis using RNA extracted from parental and

dormant SUM159 cells. Chemotherapy-enriched dormant cells exhibited increased bFGF mRNA compared to parental tumor cells (Fig. 9).

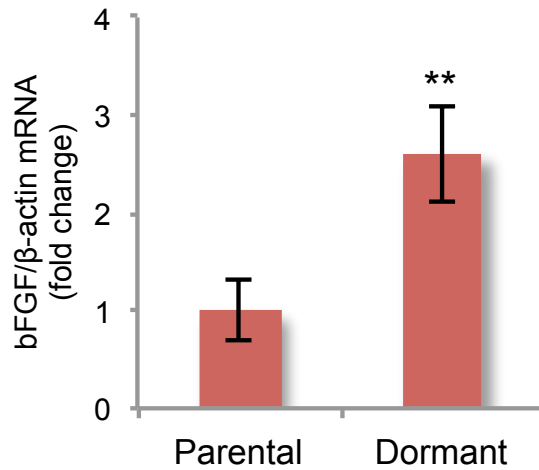


Figure 9: bFGF mRNA level in dormant tumor cells[40]. Increased bFGF mRNA in dormant SUM159 cells after doxorubicin treatment as described in Fig. 1A. Total RNA was extracted from parental and dormant cells. bFGF mRNA was quantified by qRT-PCR, and is shown as fold increase to β -actin. Error bars represent S.D., $n=3$, ** $p<0.01$, two-tailed student's t-test.

4.2.2 Chemotherapy-enriched dormant cells express increased nuclear bFGF

To elucidate the connection between different isoforms of bFGF and tumor dormancy, we measured nuclear and cytosolic bFGF levels in chemotherapy-enriched dormant tumor cells. Western blot results showed significantly increased levels of nuclear bFGF isoforms (22, 24 kD), but not the cytosolic bFGF isoform (18 kD), in dormant cells compared to parental cells (Fig. 1D). This trend was observed regardless of the chemotherapy class studied (doxorubicin or docetaxel, Fig. 10).

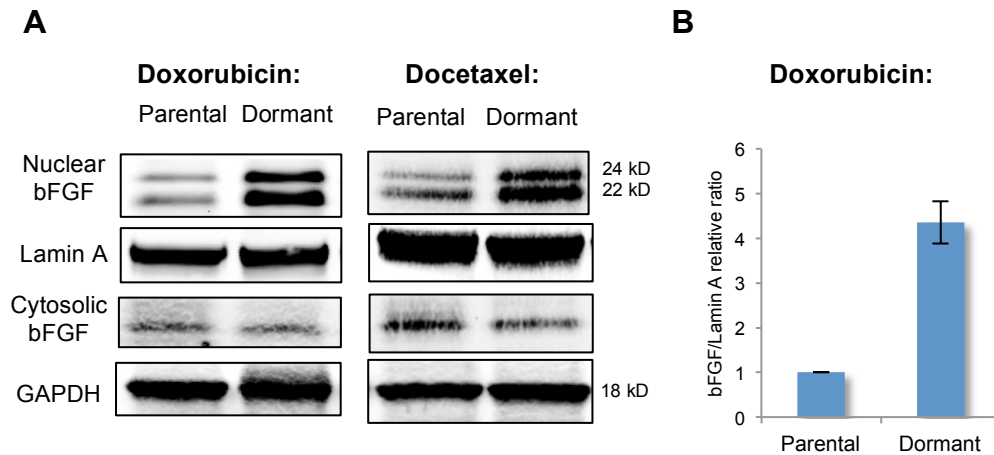


Figure 10: Dormant tumor cells express increased nuclear bFGF[40]. A. Increased expression of nuclear, but not cytosolic, bFGF in dormant SUM159 cells after doxorubicin or docetaxel treatment (as described in Fig. 1A). Nuclear or cytosolic protein was extracted from parental and dormant cells. Equivalent amounts were immunoblotted with bFGF, Lamin A, or GAPDH antibody, followed by IrDye-conjugated secondary antibodies. Protein bands were detected by infrared imaging. **B.** Protein bands from three independent trials (doxorubicin treatment, as described in Fig. 1A) were quantified using Image J software (NIH), and the relative ratio of nuclear bFGF to loading control is shown for parental and dormant SUM159 cells. Error bars represent S.D., n=3, ***p<0.001, two-tailed student's t-test.

4.2.3 Immunofluorescence for bFGF in dormant tumor cells

By immunofluorescence, we confirmed increased nuclear bFGF in dormant relative to parental TN tumor cells for both SUM159 and BT549 tumor cells (Fig. 11) as well as for two other TN breast tumor cell lines (HS578T and MDA-MB-231, data not shown). These results suggest an association of nuclear bFGF expression with TNBC dormancy following chemotherapy treatment.

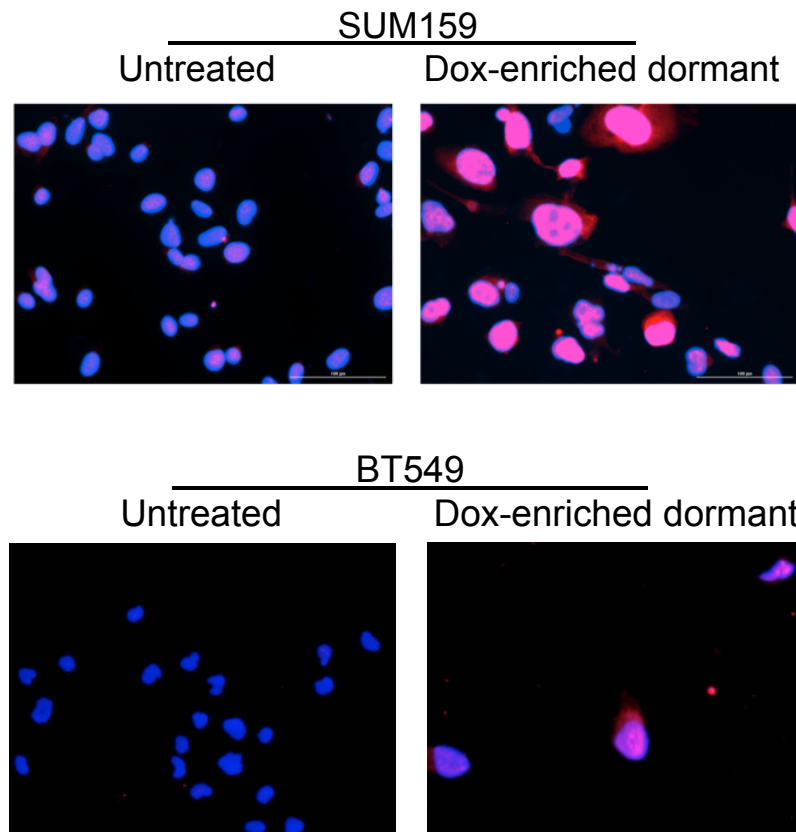


Figure 11: Immunofluorescence for bFGF in dormant tumor cells[40]. SUM159 and BT549 cells were treated with doxorubicin as described in Fig. 1A. Parental and dormant cells were fixed and stained with Hoechst (blue) and bFGF antibody (red) to demonstrate the increased nuclear localization of bFGF in dormant TN breast tumor cells. Magnification 40X.

4.2.4 bFGF knockdown in TN breast tumor cells reduces the number of chemo-enriched dormant cells

To determine whether bFGF is required for TNBC dormancy and recurrence, we knocked down bFGF expression in SUM159 and BT549 cells by stable bFGF shRNA transfection (Fig. 12A). Cells transfected with bFGF or control shRNA were treated for 2d with doxorubicin as in Fig. 1A. The number of dox-enriched dormant cells on day 7 was significantly decreased in bFGF shRNA transfectants compared to control shRNA transfectants (Fig. 12B and C).

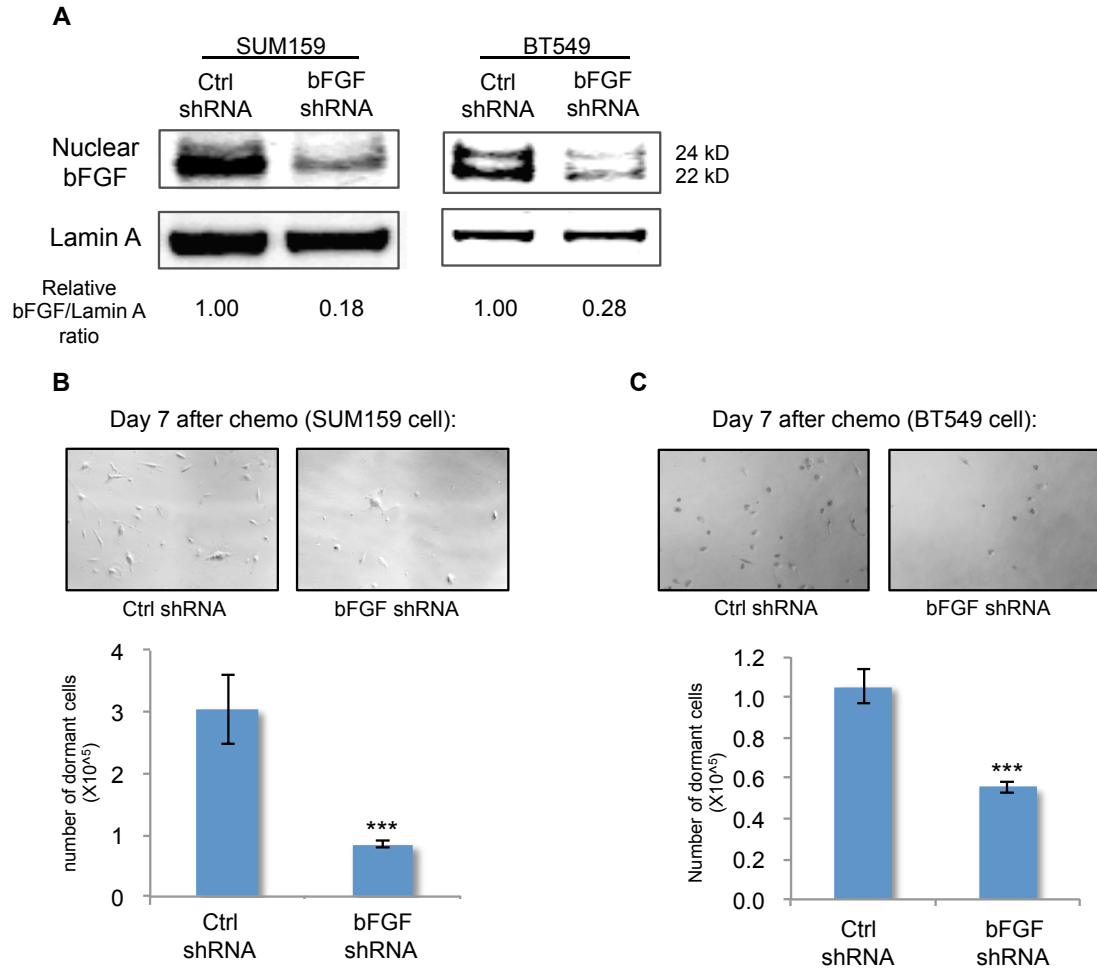


Figure 12: A. bFGF knockdown in TN breast tumor cells reduces the number of chemo-enriched dormant cells[40]. A. SUM159 and BT549 cells were transfected stably with a bFGF shRNA or control shRNA. The knockdown of nuclear bFGF was confirmed by immunoblotting equivalent amounts of nuclear extract with bFGF antibody. Protein loading was accessed using Lamin A antibody. Protein bands were quantified using Image J software (NIH), and the relative ratio of bFGF to loading control is shown for each lane. **B and C.** SUM159 cells (B) and BT549 cells (C) transfected stably with a bFGF shRNA or control shRNA were treated with doxorubicin as described in Fig. 1A. Upper panel: Pictures of remaining dormant cells were taken on d7. Magnification 20X. Lower panel: Numbers of chemotherapy-enriched dormant cells on d7 were determined by trypan blue exclusion. n=3, error bars represent S.D., ***p<0.001, two-tailed student's t-test.

4.2.5 bFGF knockdown in TN breast tumor cells decreases recurrent colony formation

To further characterize whether bFGF is required for dormant tumor cell regrowth and recurrent colony formation, we treated the above mentioned shRNA-transfected cells with doxorubicin for 2d as in Fig. 3. The numbers of recurrent colonies (>50 cells) were quantified. bFGF shRNA transfectants formed dramatically fewer recurrent colonies after chemotherapy removal than control shRNA transfectants (Fig. 13). Collectively, these results indicate that bFGF is necessary for the survival of dormant tumor cells after doxorubicin challenge and subsequent “recurrent” colony formation.

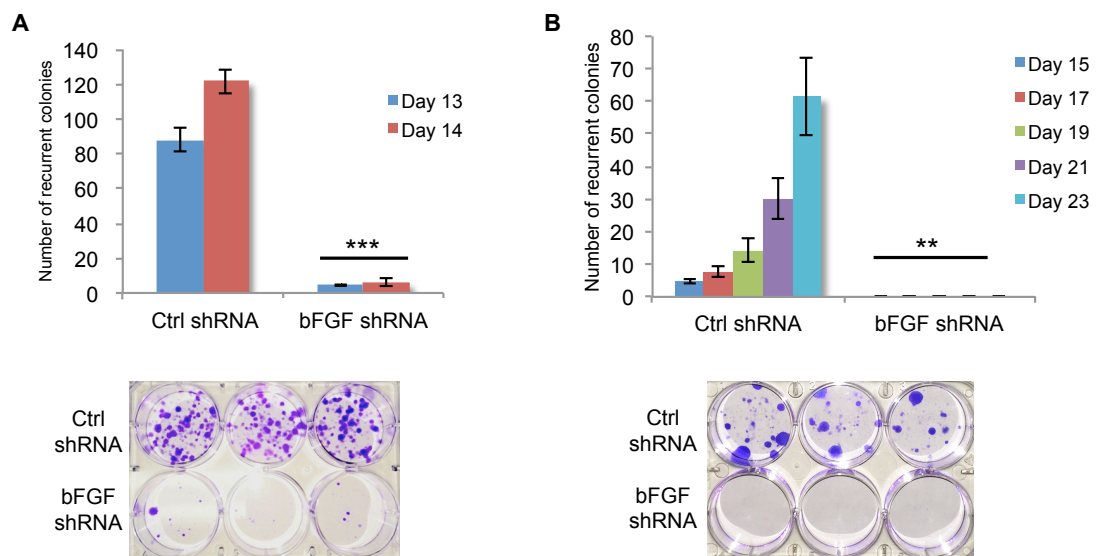
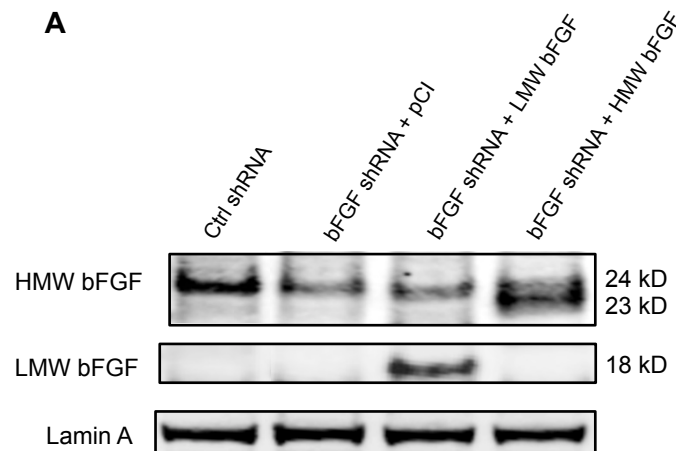


Figure 13: bFGF knockdown in TN breast tumor cells decreases recurrent colony formation[40]. SUM159 cells (**A**) and BT549 cells (**B**) transfected stably with a bFGF shRNA or control shRNA were treated with doxorubicin as described in Fig. 1A. **Upper panel:** Recurrent colonies (containing > 50 cells) were quantified on the indicated days. Error bars represent S.D., n=3, ***p<0.001, two-tailed student's t-test. **Lower panel:**

Recurrent colonies were fixed and stained with crystal violet on d22 (SUM159 cell) and d24 (BT549 cell). Similar results were obtained in at least 3 independent trials.

4.2.6 Nuclear bFGF isoform promotes the survival of chemo-residual, dormant tumor cells

The bFGF shRNA used in this study knocks down expression of both nuclear and cytosolic bFGF isoforms. To determine which bFGF isoform facilitates dormant tumor cell survival and “recurrent” colony formation in our model, we transfected bFGF shRNA-expressing cells with a vector expressing 18 kDa rat bFGF, 23 kDa rat bFGF, or an empty control vector (Fig. 14A). The 18 kDa and 23 kDa rat bFGF constructs exhibit 97% and 82% homology with human 18 kDa and 24 kDa nuclear bFGF, respectively[55]. The addback of the 23 kDa rat nuclear bFGF, but not the 18 kDa rat cytosolic bFGF, to bFGF shRNA transfectants increased the number of chemo-residual dormant tumor cells to that observed in control cells (Fig. 14B).



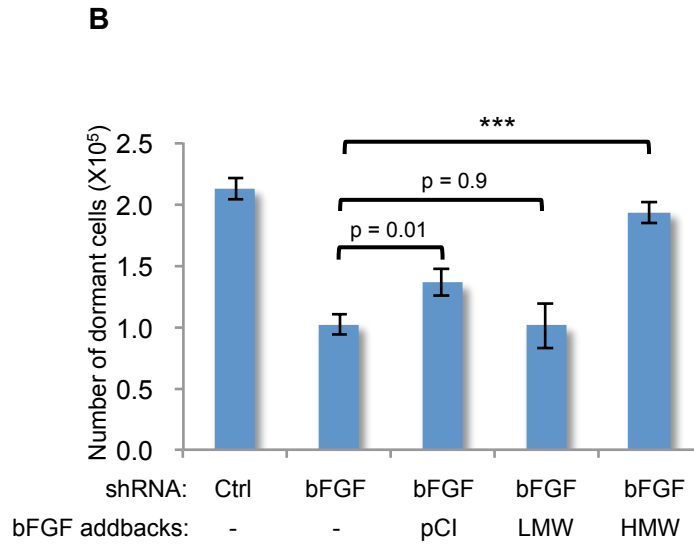


Figure 14: Transfection of bFGF knockdown cells with HMW (nuclear) bFGF vector restores dormant cell survival[40]. **A.** bFGF shRNA-transfected SUM159 cells were transfected with vectors expressing LMW (cytosolic) bFGF, HMW (nuclear) bFGF, or pCI as a vector control. The expression of addback constructs in stable transfectants was confirmed and compared to control shRNA-transfected cells by immunoblotting equivalent amounts of nuclear extract with bFGF antibody. Protein loading was assessed using Lamin A antibody. **B.** SUM159 cells expressing control shRNA, bFGF shRNA, or bFGF shRNA plus indicated addback constructs were treated with doxorubicin as described in Fig. 1A. The number of chemotherapy-enriched dormant cells was determined on d7 by trypan blue exclusion. Error bars represent S.D., n=3, ***p<0.001, two-tailed student's t-test.

4.2.7 Nuclear bFGF isoform promotes recurrent colony formation

Likewise addback of the 23 kDa bFGF isoform restored the ability of bFGF shRNA transfectants to establish recurrent colonies following short term doxorubicin treatment (Fig. 15). Collectively, these results demonstrate that high molecular weight (nuclear) bFGF, but not low molecular weight (cytosolic) bFGF, is sufficient to maintain

the viability of dormant tumor cells and promote recurrent colony growth after chemotherapy treatment.

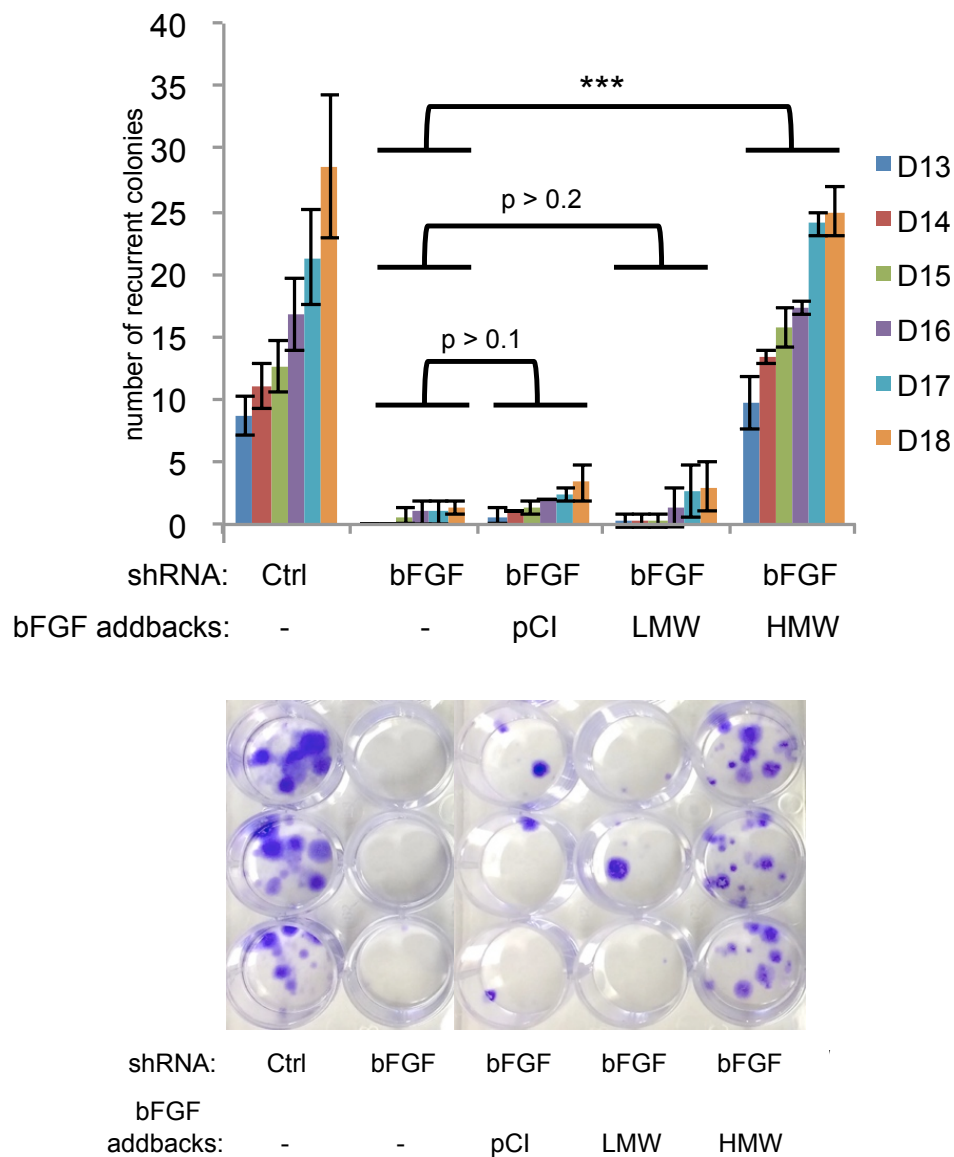


Figure 15: Transfection of bFGF knockdown cells with HMW (nuclear) bFGF vector restores recurrent colony formation[40]. **Upper panel:** Recurrent SUM159 colonies (containing > 50 cells) were quantified on the indicated days. Error bars represent S.D., n=3, ***p<0.001, two-tailed student's t-test. **Lower panel:** Recurrent colonies were fixed and stained with crystal violet on d20. Similar results were obtained in at least 3 independent trials. LMW, low molecular weight; HMW, high molecular weight.

4.2.8 Chemotherapy-enriched dormant tumor cells exhibit accelerated DNA double strand break repair

Elevated DNA repair activity is associated with chemoresistance in many tumors[29, 56-58]. To compare the DNA double strand break (DSB) repair capability, we re-challenged the untreated parental cells and chemo-enriched dormant cells with doxorubicin (DNA damaging agent) for 3 h and examined their recovery by neutral comet assay. The percentage of cells with comet tails returned to baseline faster in dormant cells than in parental cells. Similar results were observed in both SUM159 (Fig. 16A) and BT549 (Fig. 16B) dormancy models. This data indicates that chemotherapy-enriched dormant tumor cells repaired DNA double strand breaks more quickly than parental cells.

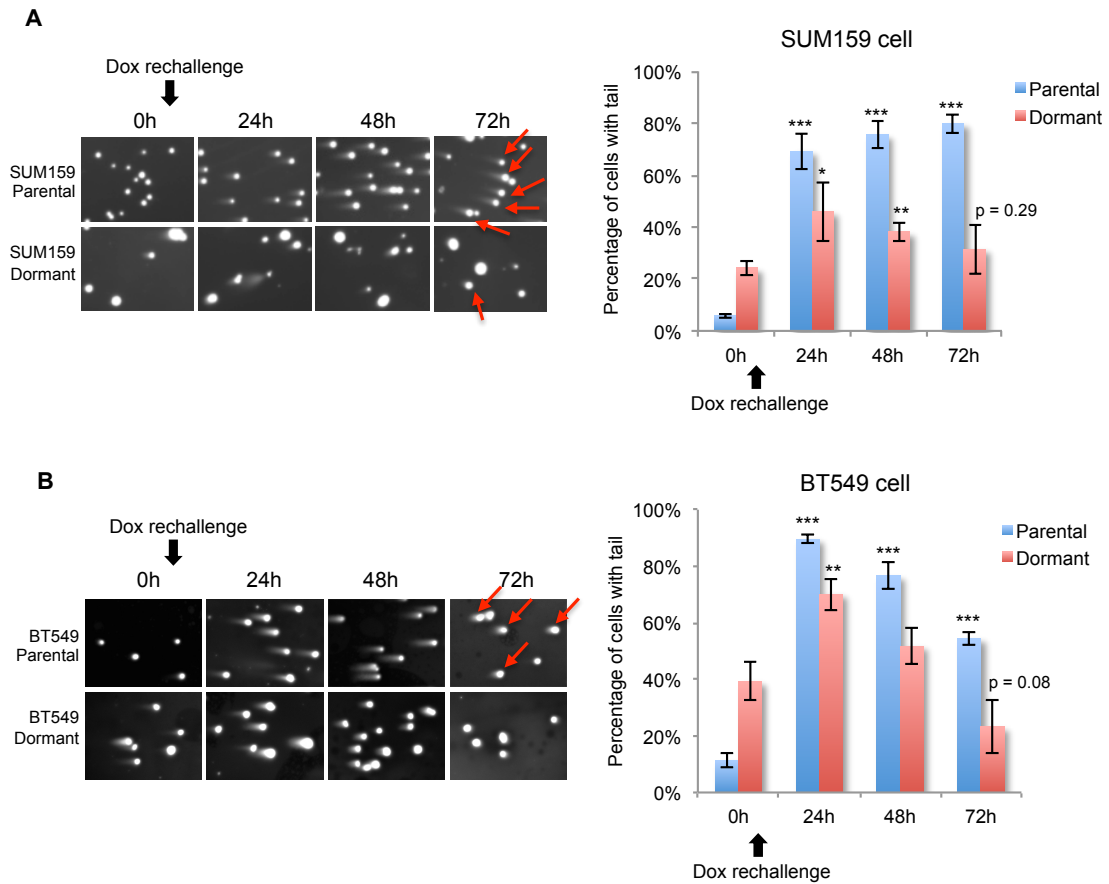


Figure 16: Dormant tumor cells have higher DNA repair capability[40]. A. Left panel: SUM159 dormant cells and parental (untreated) cells were re-challenged with doxorubicin (1 $\mu\text{g/ml}$) for 3h. Fresh medium was added after chemotherapy removal. DNA damage at sequential time points after chemotherapy treatment was analyzed by neutral comet assay. Representative images are shown at each time point. Cells scored as comet tail-positive are indicated with red arrows in the 72 h time frame. **Right panel:** The percent cells with comet tails at the indicated time points was quantified with a fluorescence microscope using Gen5 image analysis software (BioTek, VT). Error bars represent S.D., n=3 fields (each contains >50 cells). Significance of data points at 24, 48 and 72 h was determined relative to data reported at 0 h for the indicated cell population (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed student's t-test). Cells scored as comet tail-positive are indicated with red arrows in the 72 h time frame. Note that DNA damage was restored to baseline at 72 h post Dox rechallenge for dormant cells, but not for parental cells. **B. Left panel:** BT549 dormant cells and parental (untreated) cells were re-challenged with doxorubicin (0.5 $\mu\text{g/mL}$) for 4 h. DNA damage was assessed at the indicated times using the neutral comet assay as in "A". **Right panel:** The percent cells

with comet tails at the indicated time points was quantified as in “A”. Cells scored as comet tail-positive are indicated with red arrows in the 72 h time frame. Note that DNA damage was restored to baseline at 72 h post Dox rechallenge for dormant cells, but not for parental cells.

4.2.9 Chemotherapy-enriched dormant tumor cells exhibit increased DNA-PK expression/activity

DNA-dependent protein kinase (DNA-PK) is the key functional protein responsible for non-homologous end joining (NHEJ) of DNA double strand breaks (DSB). Previous studies using bFGF overexpression models suggest that nuclear bFGF drives DNA-PKcs transcription[30]. To determine whether DNA-PK activation is involved in TNBC dormancy in our *in vitro* model, we determined the expression of DNA-PKcs in chemotherapy-enriched dormant tumor cells. Dormant TN tumor cells expressed increased levels of both DNA-PKcs and phospho- Ser-2056 DNA-PK, representing the activated form of DNA-PK[27] (Fig. 17).

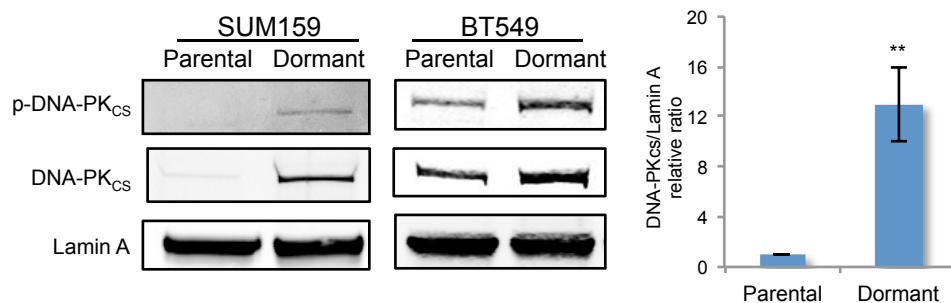


Figure 17: Dormant tumor cells have increased expression/phosphorylation of DNA-dependent protein kinase (DNA-PKcs)[40]. Left and middle panel: SUM159 cells (Left panel) and BT549 cells (Middle panel) were treated with doxorubicin as described in Fig.

1A. Nuclear protein from parental and dormant cells was extracted. Equivalent amounts were immunoblotted with phospho (Ser 2056)-DNA-PKcs and DNA-PKcs antibody. Protein loading was assessed using Lamin A antibody. **Right panel:** Protein bands from three independent trials (SUM159 cells treated with doxorubicin as described in Fig. 1A) were quantified using Image J software (NIH), and the relative ratio of DNA-PKcs to loading control is shown for each line. Error bars represent S.D., n=3, **p<0.01, two-tailed student's t-test.

4.2.10 Chemotherapy-enriched dormant tumor cells exhibit increased DNA-PK mRNA level

We next investigated DNA-PKcs mRNA levels in parental and dormant SUM159 cells by performing quantitative RT-PCR. Chemotherapy-enriched dormant cells exhibited increased DNA-PKcs mRNA expression compared to parental tumor cells (Fig. 18). Further studies are needed to determine whether the difference in mRNA level is caused by changes in transcriptional regulation or mRNA stability.

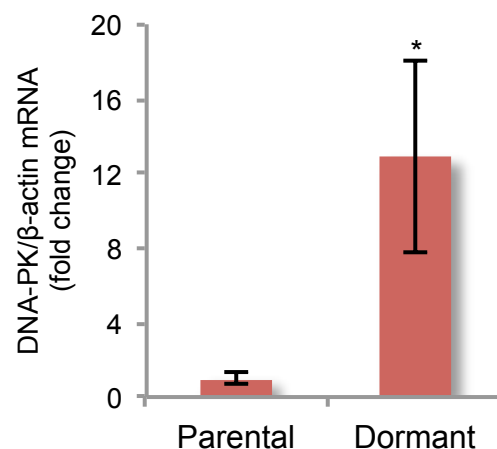


Figure 18: Dormant tumor cells exhibit increased DNA-PK mRNA [40]. SUM159 cells were treated with doxorubicin as described in Fig. 3. Total RNA from parental and dormant cells (d7) was extracted. DNA-PKcs mRNA expression was quantified by qRT-

PCR, and is shown as fold increase to β -actin. Error bars represent S.D., $n=3$, $*p<0.05$, two-tailed student's t-test.

4.2.11 bFGF regulates DNA-PK expression/activity in chemotherapy-enriched dormant tumor cells

Overexpression of bFGF in HeLa cells drives the expression and activation of DNA-PK catalytic subunit (DNA-PK_{CS}) [30]. To determine whether nuclear bFGF regulates DNA-PK signaling in TNBC dormancy in our *in vitro* model, we determined the expression of DNA-PK_{CS} in dormant bFGF knockdown cells. bFGF knockdown significantly decreased the DNA-PK_{CS} protein level in chemotherapy-enriched dormant tumor cells, indicating an upstream regulation of DNA-PK_{CS} by bFGF (Fig 19).

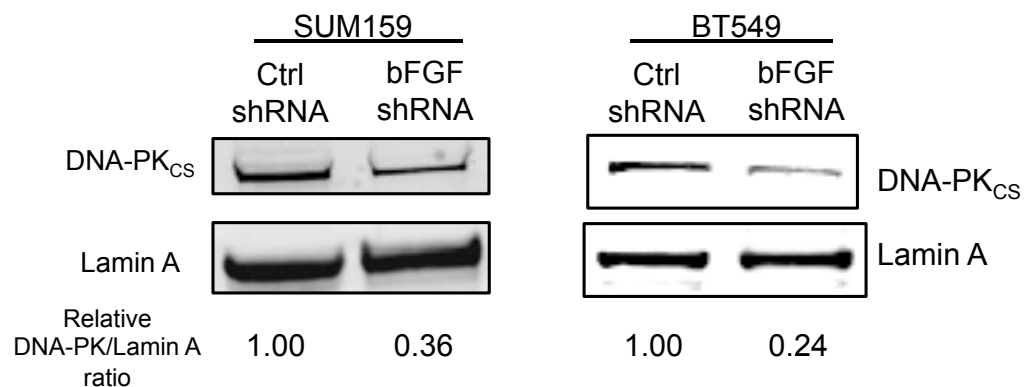


Figure 19: bFGF knockdown decreases the expression of DNA-dependent protein kinase (DNA-PK_{CS}) in dormant tumor cells[40]. SUM159 or BT549 cells transfected with bFGF shRNA or control shRNA were treated with doxorubicin as described in Fig. 1A. Nuclear protein from dormant cells was extracted. Equivalent amounts were immunoblotted with DNA-PK_{CS} antibody. Protein loading was accessed using Lamin A antibody. Protein bands were quantified using Image J software (NIH), and the relative ratio of DNA-PK_{CS} to loading control is shown for each lane.

4.2.12 bFGF knockdown in TN breast tumor cells decreases DNA double strand break (DSB) repair

To further confirm bFGF regulation of DNA repair, we examined the recovery after short term chemotherapy in bFGF or control shRNA transfectants by neutral comet assay. Twenty-four hours after doxorubicin challenge, cells expressing a bFGF shRNA showed a similar level of DNA damage to that of cells expressing a control shRNA, with approximately 70% of cells having a comet tail (Fig. 20). However, control shRNA-expressing cells exhibited more rapid DNA repair than bFGF shRNA transfectants, with only approximately 30% control shRNA-expressing cells having comet tails at 48 h post challenge (compared to approximately 60% of bFGF shRNA-expressing cells having comet tails at this time) (Fig. 20). Collectively, these results suggest that chemotherapy-enriched TN dormant tumor cells support a bFGF/DNA-PK signaling axis that confers accelerated DNA double strand break (DSB) repair capability, allowing them to survive chemotherapy challenge and establish recurrent tumors.

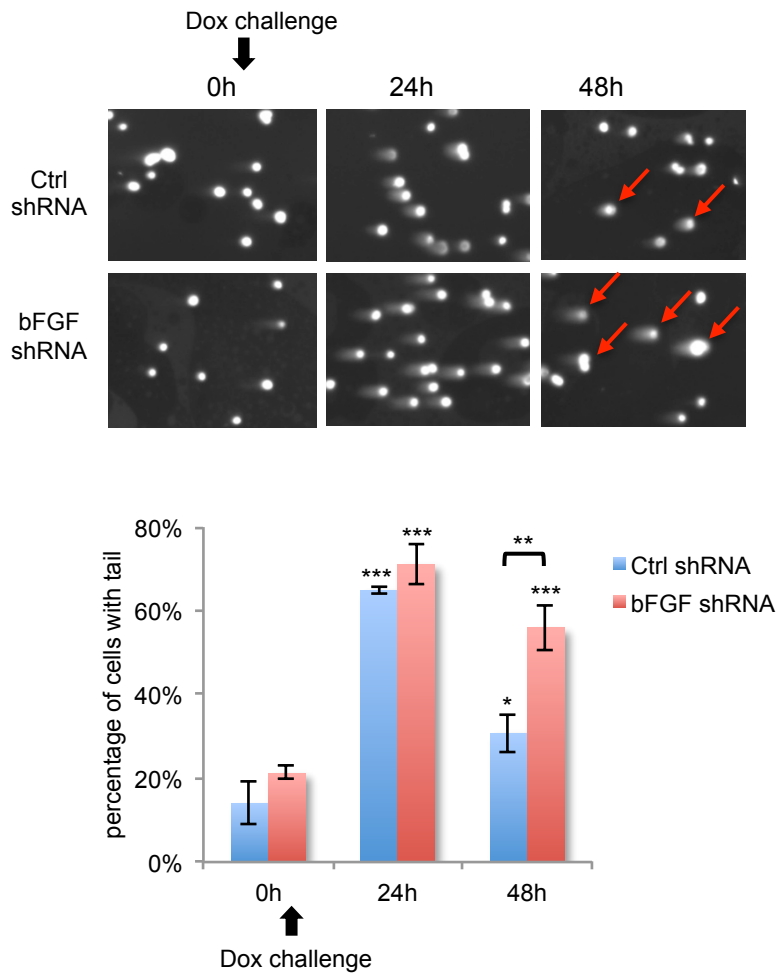


Figure 20: bFGF knockdown reduces DNA repair capability in TN breast tumor cells[40]. **Upper panel:** BT549 cells transfected with bFGF shRNA or control shRNA were challenged with doxorubicin (0.25 $\mu\text{g/ml}$) for 2 h. Fresh medium was added after chemotherapy removal. DNA damage at sequential time points after chemotherapy treatment was analyzed by neutral comet assay. Representative images are shown for each time point. Cells scored as comet tail-positive are indicated with red arrows in the 48 h time frame. **Lower panel:** The percentages of cells with comet tails at indicated time points were quantified with a fluorescence microscope using Gen5 image analysis software (BioTek, VT). Error bars represent S.D., n=3 fields (each field containing >50 cells). Significance of data points at 24 and 48 h was determined relative to data reported at 0 h for the indicated cell population (*p<0.05, **p<0.01, ***p<0.001, two-tailed student's t-test). Note that by 48 h, the percentage of cells with DNA damage was significantly reduced for control shRNA cells, but not for bFGF shRNA cells.

4.2.13 Inhibition of DNA double strand break (DSB) repair by a selective DNA-PK inhibitor decreases the survival of chemo-residual dormant TN tumor cells

NU7441 is a specific inhibitor of DNA-PK with 100-fold selectivity for DNA-PK compared to other PI3K kinase family members[26, 59]. To determine whether DNA-PK inhibition can reduce TN dormant tumor cell survival and regrowth, we simultaneously treated SUM159 TN breast tumor cells with doxorubicin and NU7441 at either of two non-cytotoxic concentrations[26]. NU7441 significantly decreased the number of doxorubicin-enriched dormant cells compared to DMSO control (Fig. 21).

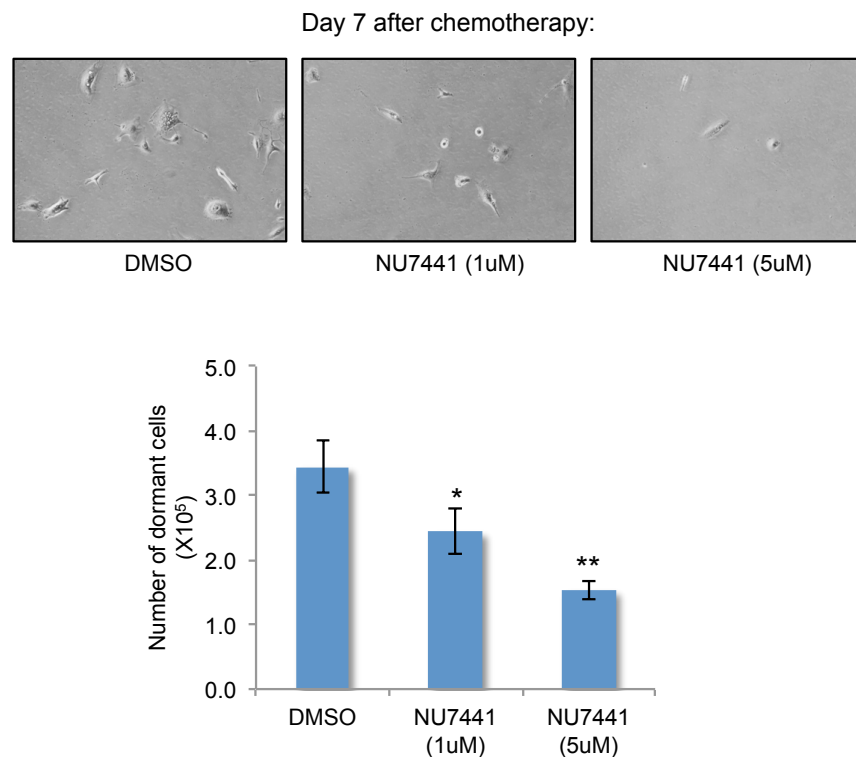


Figure 21: Selective DNA-PK inhibitor (NU7441) reduces dormant tumor cell survival[40]. SUM159 cells were treated with doxorubicin (1 μ g/ml) plus DMSO or a selective DNA-PK inhibitor (NU7441), at non-cytotoxic concentration[26] (1 μ M or 5 μ M) for 2 d, as described in Fig. 1A. Fresh medium was added after treatment removal.

Upper panel: Pictures of remaining dormant cells were taken on d7. Magnification 40X.
Lower panel: The numbers of dormant cells on d7 were determined by trypan blue exclusion. Error bars represent S.D., n=3, *p<0.05, **p<0.01, two-tailed student's t-test.

4.2.14 Inhibition of DNA double strand break (DSB) repair by a selective DNA-PK inhibitor decreases recurrent colony formation

Likewise, treating SUM159 cells with doxorubicin and NU7441 at either of two non-cytotoxic concentrations significantly decreased the number of recurrent colonies compared to DMSO control (Fig. 22). Collectively, these results suggest that DNA-PK inhibition by NU7441 in combination with chemotherapy can be a potential strategy to eliminate chemo-residual dormant TN tumor cells and block tumor recurrence.

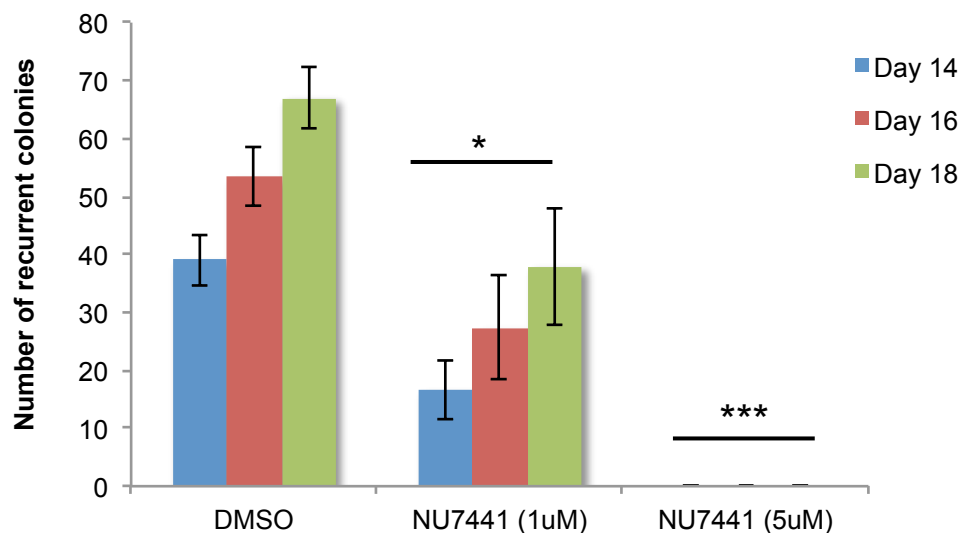


Figure 22: Selective DNA-PK inhibitor (NU7441) blocks TN breast cancer cell recurrent colony formation[40]. SUM159 cells were treated with doxorubicin (1 μ g/ml) plus DMSO or a selective DNA-PK inhibitor (NU7441), at non-cytotoxic concentration[26] (1 μ M or 5 μ M) for 2 d, as described in Fig. 1A. Fresh medium was added after treatment removal. “Recurrent” colonies (contain > 50 cells) were quantified

on d 14, 16, 18, respectively. Error bars represent S.D., n=3. Significance was determined relative to DMSO treated cells at each time point using two-tailed student's t-test (*p<0.05, ***p<0.001). Similar results were obtained in at least three independent trials.

4.2.15 bFGF expression in tumor samples from TNBC patients is associated with a higher recurrence rate

We examined an association between bFGF mRNA expression and tumor recurrence status in TNBC patients using the Oncomine cancer gene expression database. We found 30 datasets containing information on bFGF expression in TNBC, but only 1 of the 30 datasets reported recurrence status[36]. In this subset, patients who showed recurrence within 5 years of chemotherapy treatment had significantly higher bFGF mRNA expression in their tumors than patients without recurrence at 5 years (Fig. 23; p=0.03 calculated by Mann-Whitney U test). This finding suggests that bFGF expression in primary TN breast cancers is associated with increased risk of tumor recurrence.

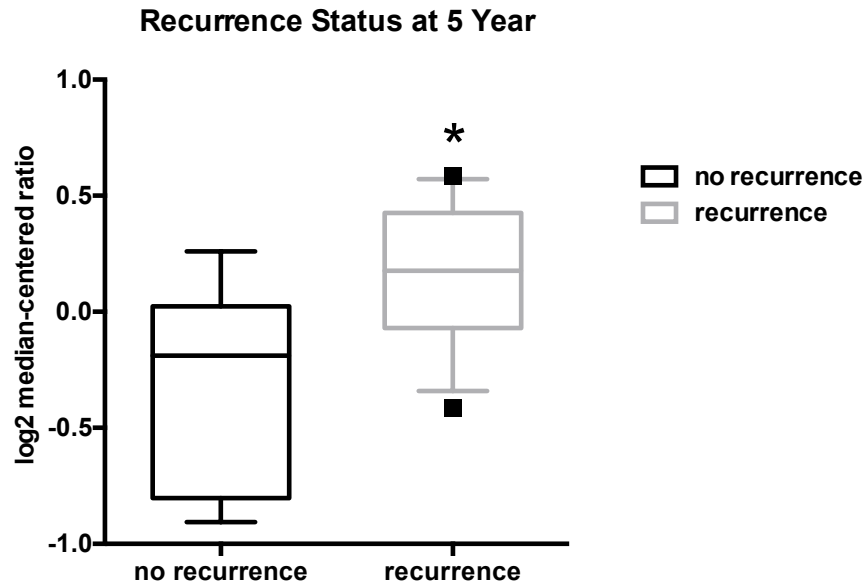


Figure 23: Upregulated bFGF expression in tumor samples from TNBC patients is associated with a higher recurrence rate after neoadjuvant chemotherapy [40]. We tested for a correlation between bFGF mRNA expression and tumor recurrence status in TNBC patients using the Oncomine cancer gene expression database. This I-SPY 1 Trial[36] was the only one which met our selection criteria, providing data on tumor recurrence. TNBC patients (n=19) received neoadjuvant chemotherapy (mRNA from tumor samples obtained prior to patient treatment were harvested for microarray analysis. bFGF expression in tumors from patients prior to treatment was compared between patients without recurrence and patients with recurrence after 5 years. Data were presented in a Box and whisker plot. The boxes span the interquartile range. The line within each box indicates the median. The ends indicate the 90th and the 10th percentile, and the dots represent the maximum and minimum outlier values. The number of patients without recurrence was 6, the number of patients with recurrence was 13, *p<0.05, Mann-Whitney U test.

4.3 Discussion

While cytosolic (secreted) bFGF has been implicated in tumor cell therapy resistance[18-23], functions for nuclear bFGF isoforms in breast cancer have not been identified. Our work is the first to implicate nuclear bFGF in TN breast cancer chemotherapy resistance/tumor recurrence. The data suggest that nuclear bFGF drives resistance to anthracycline chemotherapy, an activity that is associated with nuclear bFGF regulation of DNA-PKcs expression/DNA repair.

Previous studies indicate that tumor resistance to anti-angiogenic therapy is associated with increased expression of cytosolic FGF, which is able to restore tumor angiogenesis[18-23]. Accordingly, we were surprised to observe upregulation of nuclear bFGF isoforms, but not cytosolic bFGF isoforms, in chemotherapy-enriched dormant TN tumor cells. Our studies suggest that nuclear bFGF may drive TN breast cancer resistance in a manner independent of angiogenesis, a topic of future investigation.

We observed that chemotherapy-enriched dormant cells, relative to parental SUM159 tumor cells, express increased levels of both the 22 kDa and the 24 kDa nuclear isoforms of bFGF (Fig. 10). In order to determine whether both nuclear bFGF isoforms are critical to our model, we performed addback studies using a 23 kDa rat nuclear bFGF construct (exhibiting 82% homology with human 24 kDa nuclear bFGF). This 23 kDa bFGF isoform alone was able to restore recurrent colony formation in SUM159 cells expressing a bFGF shRNA.

Expression of nuclear vs cytosolic bFGF isoforms is determined by alternative translation pathways. Whereas cytosolic bFGF isoforms are regulated by CAP-dependent translation, nuclear bFGF isoforms are regulated by CAP-independent translation. Notably, we observed increased protein levels of nuclear but not cytosolic bFGF isoforms in chemotherapy-enriched dormant tumor cells. Our data suggests that chemo-residual TN tumor cells may support CAP-independent translation, driving expression of nuclear bFGF/DNA repair. We are currently addressing this important hypothesis. Ultimately, it may be possible to eliminate these chemo-residual dormant tumor cells by targeting the CAP-independent translation pathway.

We also observed that bFGF RNA levels are increased in chemo-residual dormant TN tumor cells relative to parental tumor cells (Fig. 9). Identifying signaling pathways that drive bFGF transcription/mRNA stability in chemotherapy-enriched dormant cells has the potential to identify rational methods for targeting these chemo-residual tumor cells. Of note, previous studies indicate that hypoxia drives bFGF transcription in a hypoxia-inducible factor-1 (HIF-1)-dependent manner[60]. However, we did not see HIF-1 expression elevated in chemotherapy-enriched dormant cells (data not shown), suggesting an alternative driver of bFGF mRNA expression in chemo-residual tumor cells.

Our work identifies DNA-PKcs as a downstream target of nuclear bFGF in chemo-residual dormant TN tumor cells. DNA-PKcs has previously been implicated in

therapy resistance[29, 56, 57, 61]. Long term chemotherapy selection models have been shown to select for chemo-resistant tumor cells with increased DNA-PKcs expression/activity[61, 62]. The current study is unique in identifying a nuclear growth factor (nuclear bFGF) in chemo-residual tumor cells that drives DNA-PKcs expression. Based on the previous demonstration that nuclear bFGF over-expression drives DNA-PKcs transcription[51], we are currently addressing the hypothesis that nuclear bFGF in chemo-residual TN tumor cells drives DNA-PKcs transcription.

We also show that a small molecule inhibitor of DNA-PKcs reduces both the number of chemo-residual, dormant tumor cells (Fig. 21) and the number of recurrent colonies (Fig. 22) in our *in vitro* model of TNBC recurrence. This activity may be attributed to multiple reported activities of DNA-PK. This DNA-PKcs inhibitor likely reduces the number of chemo-residual dormant tumor cells by blocking DNA repair, leading to increased tumor cell apoptosis. However, this inhibitor may also chemo-sensitize TN tumor cells by inhibiting a recently reported, non-conventional activity for DNA-PK, namely its ability to induce AKT-dependent cell survival[63]. Finally, DNA-PK is a critical regulator of mitosis[64]. Thus it is possible that inhibiting DNA-PK in our model prevents the transition of chemo-residual dormant tumor cells to proliferative “recurrent” tumor cells.

Nuclear-localized EGF receptor is a critical determinant of DNA-PK activity[65]. Based on this knowledge, in addition to our current findings, it is intriguing to speculate

that nuclear bFGF may control DNA-PKcs expression/activity in a manner dependent on a nuclear bFGF receptor, a topic of current investigation. This possibility is supported by a literature demonstrating that nuclear FGF cooperates with a nuclear FGF receptor to drive gene transcription in neurons[66]. Identifying a bFGF receptor that drives nuclear bFGF/DNA-PK signaling has the potential to define a logical therapeutic strategy for eliminating chemo-residual triple-negative breast cancer cells, thus preventing tumor recurrence.

In summary, using an *in vitro* model, we demonstrate a critical role for nuclear bFGF in TNBC chemotherapy resistance/tumor recurrence. To begin to determine the relevance of these findings in the clinic, we also analyzed gene expression profiles of triple-negative breast cancer patients. Notably, we found that bFGF expression in primary TN breast cancers is associated with tumor recurrence (Fig. 23). Future studies are needed to determine which bFGF isoforms (nuclear or cytosolic) are associated with tumor recurrence in TN breast cancer patients. Based on our demonstration that chemotherapy enriches for bFGF-expressing tumor cells *in vitro*, it will also be important to examine frequency of bFGF protein expression in residual tumor cells from neoadjuvant chemotherapy-treated TN breast cancer patients.

5. “Recurrent” tumor cells evolving from *in vitro* model of TN Breast Cancer dormancy/ recurrence exhibit increased therapy resistance/invasive behavior

5.1 Introduction

Tumor recurrence is the principle cause of death in TN breast cancer patients. In the clinical setting, after the diagnosis of TN breast cancer, patients often receive neoadjuvant chemotherapy treatment, followed by surgical intervention and adjuvant chemotherapy. Although achieving high rates of pathologic complete response (pCR), TN breast cancer patients have high rates of local or distal tumor recurrence. The recurrent tumors are often refractory to different classes of chemotherapy and are usually lethal to the patients[3, 67, 68].

Using short term chemotherapy to resemble clinical neoadjuvant chemotherapy, we have developed an *in vitro* model of tumor recurrence as described above in section 3. In this model, about two weeks after chemotherapy removal, chemotherapy-enriched dormant tumor cells resume growth and establish “recurrent” colonies that consist of proliferating cells. These proliferating “recurrent” cells exhibit resistance to the original chemotherapy as well as cross-resistance to a different class of chemotherapy. Moreover, they show increased DNA repair capacity and DNA-PK expression/activity that protect cells from DNA-damaging chemotherapeutic agents. Furthermore, recurrent tumor cells demonstrate accelerated migration and invasion ability. Such features are consistent with the aggressive biological behavior of recurrent tumors in TN breast cancer patients.

Thus, the characterization of recurrent tumor cells in our *in vitro* model and the exploration of the underlying mechanisms may provide potential targets for developing effective therapies against TN breast cancer recurrence.

5.2 Results

5.2.1 Recurrent tumor cells are more resistant to chemotherapy than parental tumor cells

Recurrent tumors are frequently detected in cancer patients many years after initial chemotherapy treatment, and these tumors are chemo-refractory[69]. Similar to recurrent tumors in patients, recurrent tumor cells evolving in our model from chemotherapy-enriched dormant cells exhibited increased chemotherapy resistance (Fig. 24). Increased therapy resistance was observed in two-independent recurrent breast tumor cell lines (Rec-511 and Rec-817tx). Notably, resistant recurrent breast tumor colonies were observed independent of the class of chemotherapy treatment (taxane vs anthracycline) (Fig. 24).

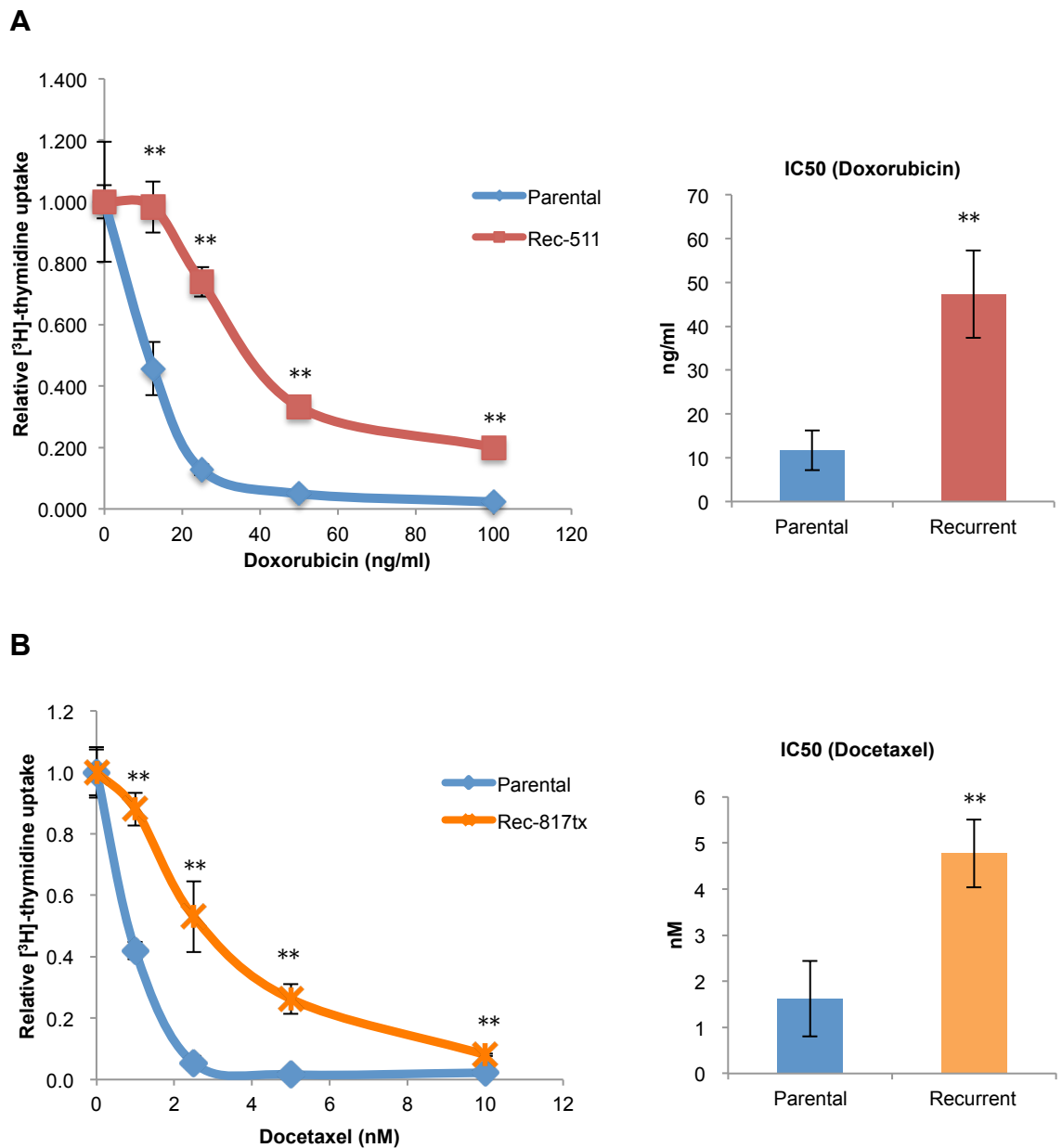


Figure 24: Tumor cells from recurrent colonies are more resistant to chemotherapy than parental tumor cells[41]. A. Left panel: SUM159 breast tumor cells were incubated with doxorubicin as in Fig. 3. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies (Rec-511), as well as parental tumor cells, were re-challenged with the indicated concentrations of doxorubicin. Chemo-sensitivity was assessed by $[^3\text{H}]$ -thymidine incorporation. Data for each point are expressed as fold change relative to cells cultured in medium only. **Right panel:** IC_{50} was calculated using linear regression analysis. Error bars represent S.D., $n=3$, $**p<0.01$, two-tailed student’s t-test. **B. Left**

panel: SUM159 breast tumor cells were incubated with docetaxel as in Fig. 4. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies (Rec-817tx) and parental tumor cells were re-challenged with the indicated concentrations of docetaxel. Chemo-sensitivity was assessed by thymidine incorporation, as in A. **Right panel:** IC₅₀ was calculated using linear regression analysis. Error bars represent S.D., n=3, **p<0.01, two-tailed student’s t-test.

5.2.2 Recurrent tumor cells have cross-resistance to different class of chemotherapy

To determine whether recurrent tumor cells evolving in our model from chemotherapy-enriched dormant cells exhibited cross-resistance to different classes of chemotherapy, we treated doxorubicin-resistant recurrent tumor cells (Rec-1217) with docetaxel, and docetaxel-resistant recurrent tumor cells (Rec-817tx) with doxorubicin. Both recurrent cell lines exhibit cross-resistance to different chemotherapy class, compared to untreated parental tumor cells (Fig. 25).

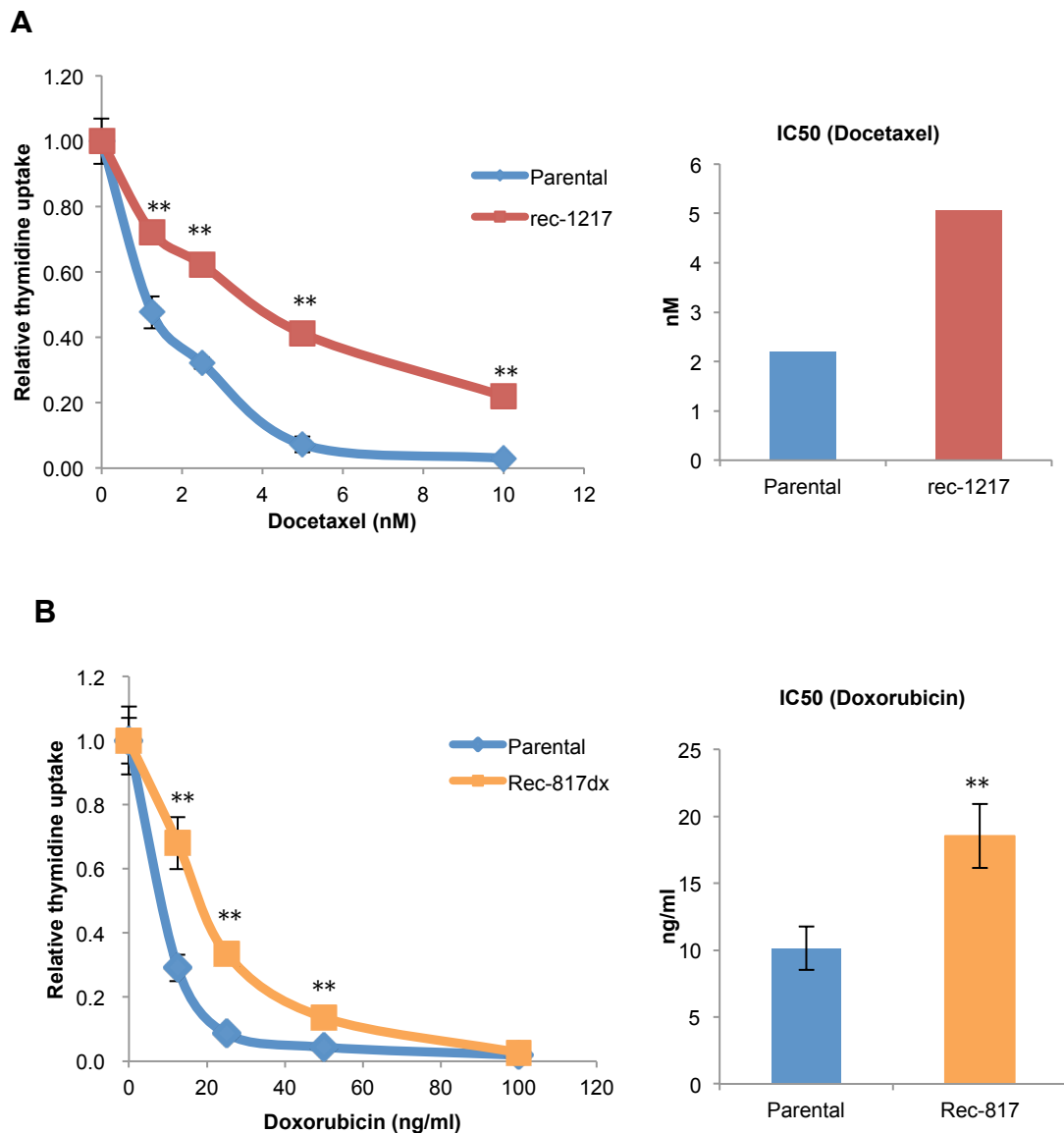


Figure 25: Recurrent tumor cells have cross-resistance to different class of chemotherapy. A. Left panel: SUM159 breast tumor cells were incubated with doxorubicin as in Fig. 3. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies (Rec-511), as well as parental tumor cells, were challenged with the indicated concentrations of docetaxel. Chemo-sensitivity was assessed by thymidine incorporation. Data for each point are expressed as fold change relative to cells cultured in medium only. Error bars represent S.D., $n=3$, $**p<0.01$, two-tailed student’s t-test. **Right panel:** IC₅₀ was calculated using linear regression analysis. **B. Left panel:** SUM159 breast tumor cells were incubated with docetaxel as in Fig. 4. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of

“recurrent” colonies. Tumor cells from recurrent colonies (Rec-817tx) and parental tumor cells were challenged with the indicated concentrations of doxorubicin. Chemosensitivity was assessed by thymidine incorporation, as in A. **Right panel:** IC50 was calculated using linear regression analysis. Error bars represent S.D., n=3, **p<0.01, two-tailed student’s t-test.

5.2.3 Recurrent tumor cells exhibit accelerated DNA double strand break (DSB) repair

We showed in section 4.2.8 that chemotherapy-enriched dormant tumor cells exhibit accelerated DNA double strand break repair. To examine the DNA double strand break (DSB) repair capability in recurrent tumor cells, we re-challenged the untreated parental cells and recurrent tumor cells with doxorubicin (DNA damaging agent) for 3 h and examined their recovery by neutral comet assay. As shown in Fig. 26, the percentage of cells with comet tails returned to baseline faster in recurrent cells than in parental cells. This data indicates that recurrent tumor cells repaired DNA double strand breaks more quickly than parental cells, which contributes to chemoresistance.

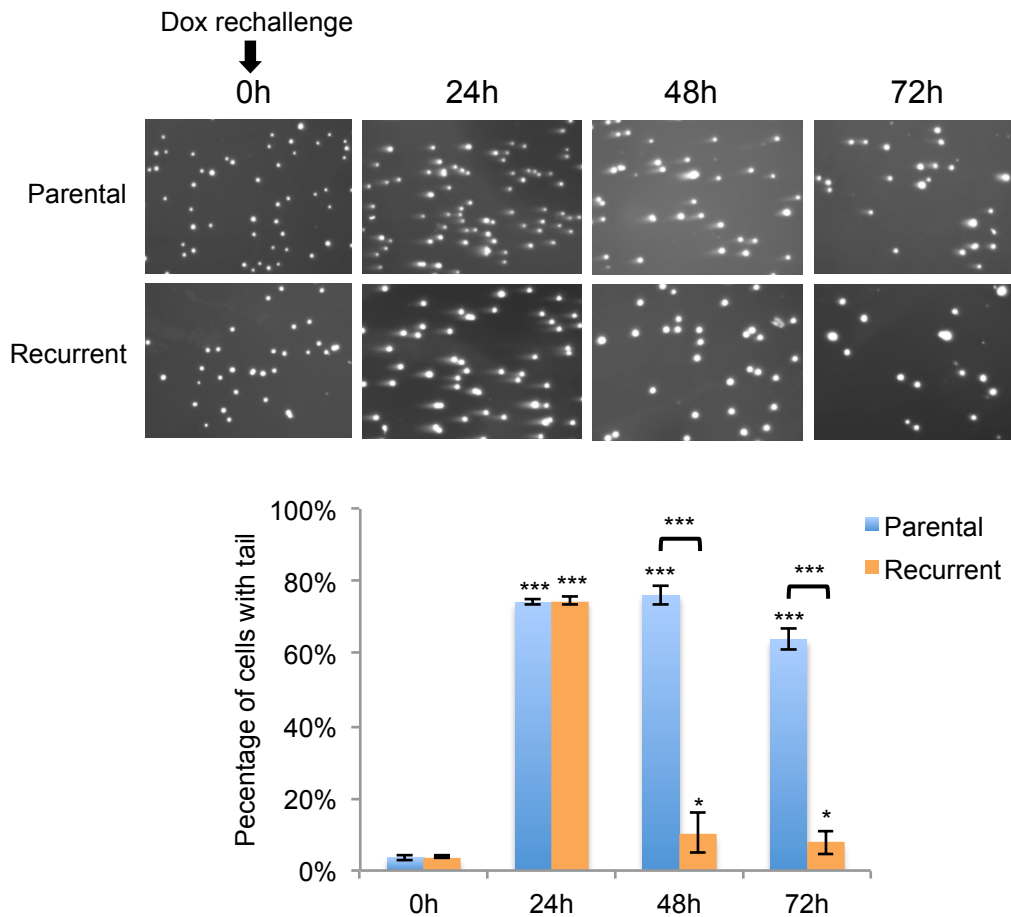


Figure 26: Recurrent tumor cells exhibit accelerated DNA double strand break repair. SUM159 were treated with doxorubicin as described in Fig. 3. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. **Upper panel:** recurrent cells and parental (untreated) cells were re-challenged with doxorubicin (1 μ g/ml) for 3h. Fresh medium was added after chemotherapy removal. DNA damage at sequential time points after chemotherapy treatment was analyzed by neutral comet assay. Representative images are shown at each time point. **Lower panel:** The percent cells with comet tails at the indicated time points was quantified with a fluorescence microscope using Gen5 image analysis software (BioTek, VT). Error bars represent S.D., n=3 fields (each contains >50 cells). Significance of data points at 24, 48 and 72 h was determined relative to data reported at 0 h for the indicated cell population (*p<0.05, **p<0.01, ***p<0.001, two-tailed student’s t-test). Note that DNA damage was significantly reduced at 48 h and 72 h post doxorubicin rechallenge for recurrent cells, but not for parental cells.

5.2.4 Recurrent tumor cells demonstrate increased DNA-PK expression/ activity

We showed in section 4.2.9 that chemotherapy-enriched dormant tumor cells exhibit increased expression/activation of DNA-PK. To determine whether DNA-PK activation is involved in chemoresistance of recurrent tumor cells, we determined the expression of DNA-PKcs in recurrent tumor cells evolving from chemotherapy-enriched dormant cells. Recurrent tumor cells expressed increased levels of both DNA-PKcs and phospho- Ser-2056 DNA-PK, representing the activated form of DNA-PK[27] (Fig. 27). Notably, Histone H2A.X (DNA damage marker) phosphorylation is decreased in recurrent cells, indicating accelerated DNA damage repair.

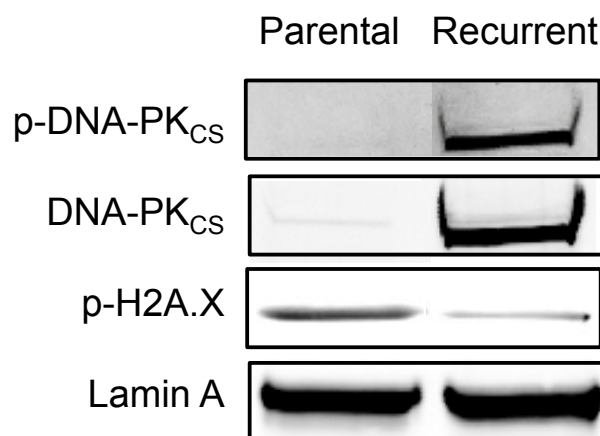


Figure 27: Recurrent tumor cells have increased expression/phosphorylation of DNA-dependent protein kinase (DNA-PKcs). SUM159 were treated with doxorubicin as described in Fig. 3. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Nuclear protein from parental and recurrent cells was extracted. Equivalent amounts were immunoblotted with phospho (Ser 2056)-DNA-PKcs, DNA-PKcs, and phospho-H2A.X antibody. Protein

loading was assessed using Lamin A antibody. Similar results were obtained in at least three independent trials.

5.2.5 Recurrent tumor cells exhibit decreased proliferation compared to parental tumor cells

To further characterize the difference in biological properties between recurrent and parental tumor cells, we determined their proliferation rate by [³H]-thymidine uptake assay. We observed significantly decreased growth rate in recurrent tumor cells compared to untreated parental cells, regardless of the chemotherapy class studied (doxorubicin or docetaxel, Fig. 28).

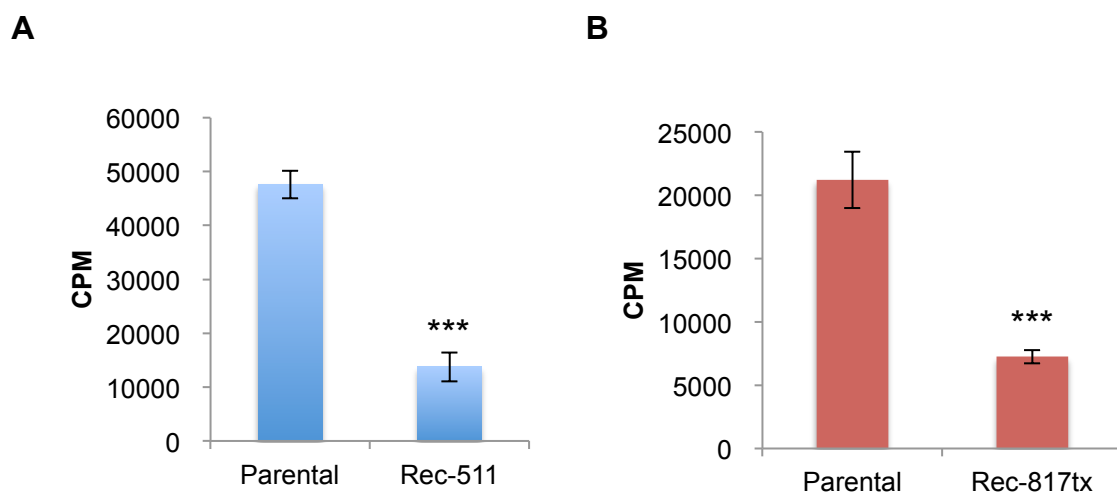


Figure 28: Recurrent tumor cells exhibit decreased proliferation. A. SUM159 breast tumor cells were incubated with doxorubicin as in Fig. 3. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies (Rec-511) as well as untreated parental tumor cells were plated to 96-well plate. Proliferation was determined by [³H]-

thymidine incorporation (+/-SD). **B.** SUM159 breast tumor cells were incubated with docetaxel as in Fig. 4. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies (Rec-817tx) as well as untreated parental tumor cells were plated to 96-well plate. Proliferation was determined by [³H]-thymidine incorporation (+/-SD). n=4, ***p<0.001, two-tailed student’s t-test.

5.2.6 Recurrent tumor cells exhibit increased migration *in vitro*

Recurrent breast tumors are frequently found in distal organs including lungs, bones, brain, and liver. Such metastasis is associated with increased migration and invasion ability of recurrent tumor cells[35]. To determine whether the recurrent tumor cells evolving in our model from chemotherapy-enriched dormant cells exhibited increased migratory capacity, we performed a wound-healing migration assay. Recurrent tumor cells demonstrated accelerated wound closure compared to untreated parental cells, indicating increased migratory capacity (Fig. 29).

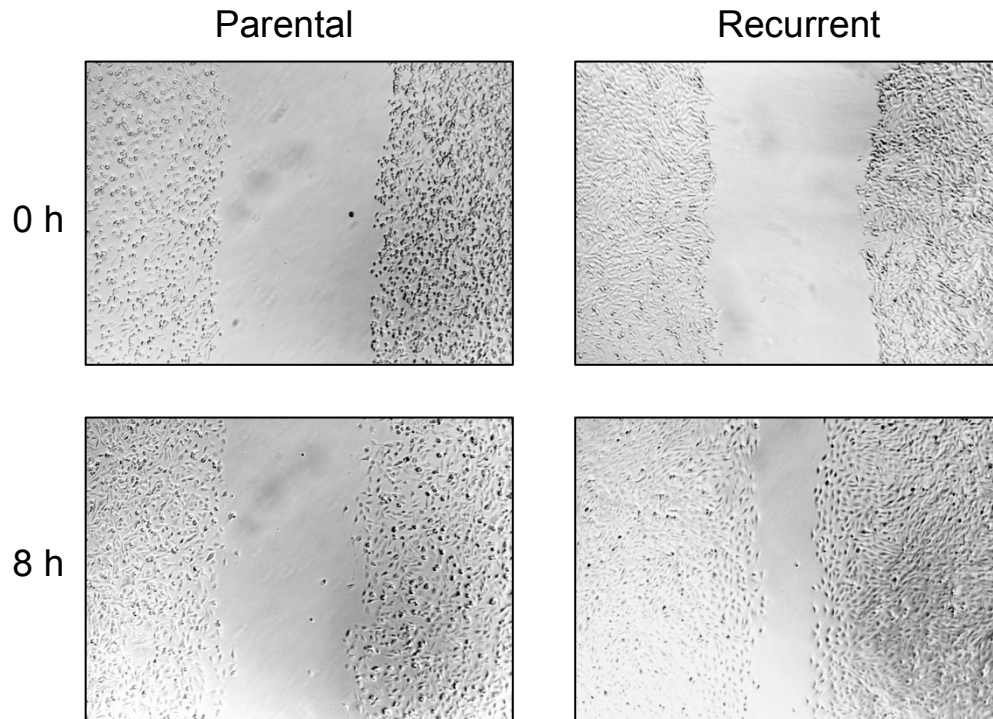


Figure 29: Recurrent tumor cells exhibit increased migration *in vitro*. SUM159 breast tumor cells were incubated with doxorubicin as in Fig. 3. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies, as well as parental tumor cells, were plated on a 24-well plate and grew to reach confluence. Cells in the middle of each well were mechanically scratched using a pipette tip. Cell debris were removed by PBS wash. Fresh medium was added. Images were taken on 0 h and 8 h. At least three random locations were examined and representative pictures were shown.

5.2.7 Recurrent tumor cells demonstrate increased invasiveness *in vitro*

To determine whether the recurrent tumor cells evolving in our model from chemotherapy-enriched dormant cells exhibited increased invasiveness, we performed a transwell invasion assay. Recurrent tumor cells migrated through matrigel faster than

untreated parental cells, indicating increased invasiveness and metastatic potential (Fig. 30).

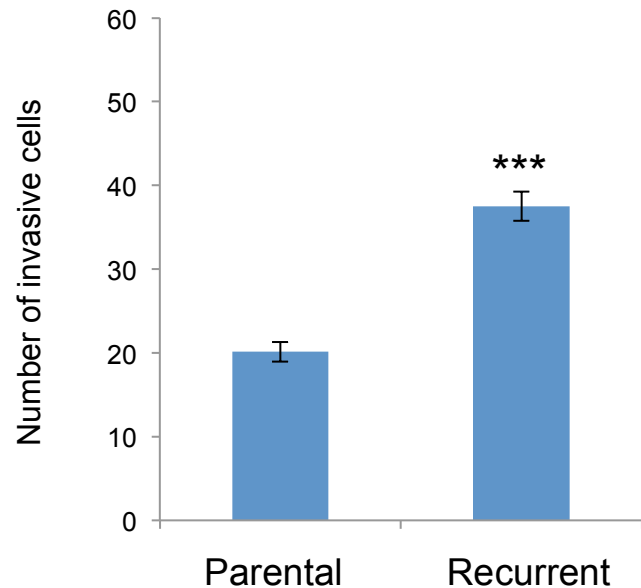


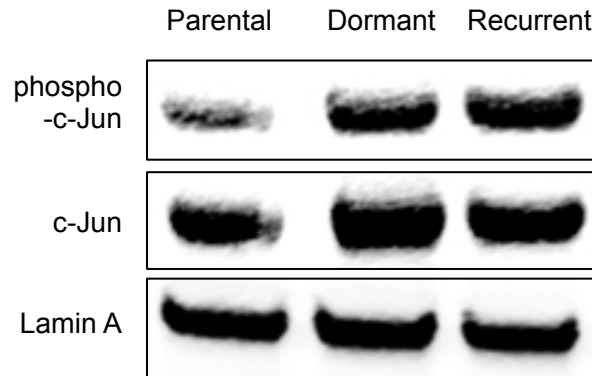
Figure 30: Recurrent tumor cells exhibit increased invasiveness *in vitro*. SUM159 breast tumor cells were incubated with docetaxel as in Fig. 4. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Tumor cells from recurrent colonies, as well as parental tumor cells, were serum-starved for 24 h. Equal amounts of cells were added to the upper chambers of a matrigel-coated plate. Complete medium was added to the bottom chambers. After 12 h, cells that migrated into the bottom chambers were fixed and stained with crystal violet. The number of migrated cells was counted from three fields under microscope. Error bars represent S.D., n=3, ***p<0.001, two-tailed student’s t-test.

5.2.8 Recurrent tumor cells exhibit increased expression of tumor migration and invasion markers

To explore the underlying mechanisms of increased migration and invasiveness in recurrent tumor cells, we examined the expression of protein markers associated with tumor migration and invasion. c-Jun is a proto-oncogene and its phosphorylation is

associated with invasive breast carcinoma. MMP-2 belongs to the matrix metalloproteinase family and is responsible for disrupting basement membrane and facilitating cancer cell migration and metastasis[70, 71]. The recurrent tumor cells evolving in our model from chemotherapy-enriched dormant cells expressed increased levels of phospho-c-Jun and MMP-2 compared to untreated parental tumor cells (Fig. 31), consistent with increased migration and invasiveness observed in wound-healing and invasion assays (Figs 29 and 30).

A



B

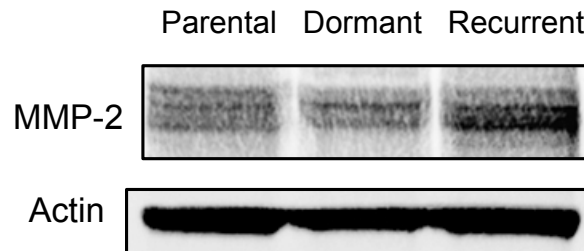


Figure 31: Recurrent tumor cells exhibit increased expression/activation of c-Jun and MMP-2. SUM159 breast tumor cells were incubated with docetaxel as in Fig. 4. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Total cellular protein was extracted from parental, dormant, and recurrent SUM159 cells, and equivalent amounts were immunoblotted with indicated antibodies, followed by IrDye-conjugated secondary antibody. Protein loading was assessed using Actin or Lamin A antibodies.

5.2.9 Recurrent tumor cells exhibit advanced Epithelial-mesenchymal transition (EMT)

Epithelial-mesenchymal transition (EMT) is a critical step for cancer migration, invasion, and metastasis. It is associated with loss of epithelial protein markers, such as pan-cytokeratin, and with gain of mesenchymal protein markers, such as N-cadherin[34,

72]. We compared the expression of EMT protein markers between parental and recurrent tumor cells in our *in vitro* model. SUM159 cell line has undergone EMT. However, the recurrent cells expressed higher N-cadherin and lower pan-cytokeratin than parental cells, indicating an advanced stage of EMT, predicting increased invasiveness and metastatic potential (Fig. 32).

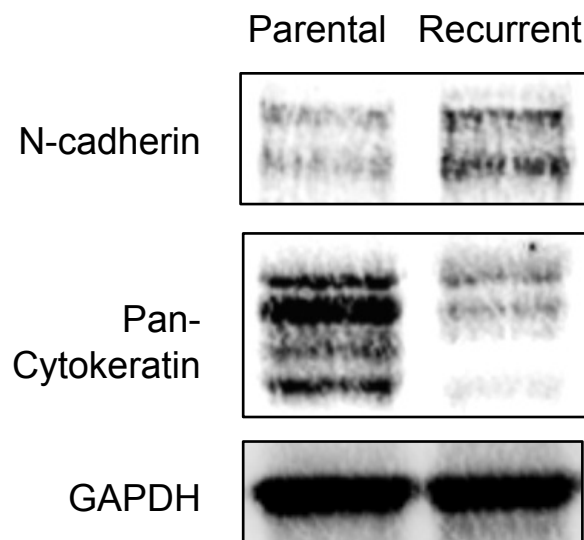


Figure 32: Recurrent tumor cells exhibit increased expression of N-cadherin and decreased expression of pan-cytokeratin. SUM159 breast tumor cells were incubated with docetaxel as in Fig. 4. Residual tumor cells were allowed to grow in the absence of chemotherapy, resulting in the evolution of “recurrent” colonies. Total cellular protein was extracted from parental and recurrent cells, and equivalent amounts were immunoblotted with indicated antibodies, followed by IrDye-conjugated secondary antibody. Protein loading was assessed using Actin or Lamin A antibodies.

5.3 Discussion

Our study focused on the ability of the TN dormant tumor cells in our short-term chemotherapy treatment model to resume growth and establish “recurrent” colonies upon chemotherapy withdrawal, resembling the process of tumor recurrence. Notably, recurrent tumor cells evolving after chemotherapy withdrawal were more resistant to subsequent chemotherapy challenge than parental tumor cells. Recurrent cells were resistant both to the original chemotherapy class, as well as to other chemotherapy classes, indicating broad chemoresistance across different regimens. The therapy resistance of recurrent tumor cells in our model resembles expanded therapy resistance of recurrent tumors in cancer patients[73]. Such resistance of recurrent cells to DNA-damaging agent (doxorubicin) can be explained by their increased expression/activation of DNA-PK and elevated DNA repair capacity. Studies have shown that microtubule-modulating agent (Paclitaxel) causes DNA damage in cancer cells by increasing reactive oxygen species (ROS)[74]. Therefore, the increased DNA repair ability in recurrent cells evolving after doxorubicin treatment may explain their cross-resistance to docetaxel. However, the specific type of DNA damage (double strand break vs. single strand break) caused by taxanes remains to be clarified, and further studies (DNA-PK knockdown, selective DNA-PK inhibitor) are needed to confirm the exact role of DNA-PK in cross-resistance to therapy. To date, we haven’t examined the expression of DNA-PK in recurrent cells evolving from docetaxel treatment. Whether they possess the same

mechanism of chemoresistance as recurrent cells evolving from doxorubicin remains to be elucidated. Additionally, we observed decreased proliferation in recurrent tumor cells. This alteration may also contribute to their resistance as most chemotherapeutic agents target fast proliferating cells[75].

The resistant phenotype of recurrent tumor cells evolving from our chemotherapy-enriched dormant cells contrasts with the reversible-resistant phenotype of tumor cells subjected to long-term drug selection[38, 47]. We have observed continued resistance of our “recurrent” breast tumor lines at least 50 days after chemotherapy withdrawal (representing approximately 40 doubling times for these cells; data not shown). The irreversible resistance of these drug resistant tumor cells has important implications for patient treatment. Specifically, the existence of irreversible drug resistant phenotypes in the original tumor argues against models suggesting that recurrent tumors arising in patients after a gap in treatment (“drug holiday”) may benefit from retreatment with the same therapy[73]. Studies are ongoing to determine if “recurrent” tumor cells from our in vitro model remain chemo-refractory for months after therapy withdrawal.

Moreover, recurrent tumor cells exhibit more aggressive behaviors compared to parental tumor cells, as demonstrated by increased migration, invasion, and EMT. Increased aggressive behaviors of recurrent cells (compared to parental cells) are supported by expression levels of molecular markers of these behaviors (e.g. N-cadherin,

MMP-2). Studies are ongoing to identify signaling pathways that can be used as potential targets for developing novel therapies that can prevent recurrent tumor growth.

Many studies suggest that tumor cells disseminated to distal organs undergo mesenchymal-epithelial transition (MET) before establishing recurrent tumors[72, 76, 77]. Surprisingly, we observed advanced EMT in recurrent cells in our model. One explanation is that the “recurrent” cells in our model do not represent the metastatic recurrent tumor cells in patients. Instead, they are more similar to the mobilizing, invasive, and mesenchymal-like tumor cells that escape into blood circulation. This similarity is supported by recent studies that the presence of circulating tumor cells (CTCs) predicts metastatic recurrence and survival in breast cancer patients, and CTCs in patients with metastatic breast cancer express both epithelial and mesenchymal molecular markers[78-80]. Accordingly, it remains possible that our *in vitro* tumor recurrence model resembles an early stage of tumor metastasis/recurrence. Specifically, chemoresistant dormant tumor cells remain quiescent during chemotherapy onslaught, but resume proliferation during interval between chemotherapy cycles (“drug holidays”), resulting in producing highly aggressive tumor cells (“recurrent” cells in our model) that later go through the invasion-metastasis cascade. Notably, our preliminary *in vivo* experiments show that after entering blood circulation through tail vein injection in mice, the recurrent cells from our *in vitro* model develop lung and bone colonization

faster and larger than untreated parental tumor cells, indicating elevated extravasation capacity and invasiveness. Studies are ongoing to examine whether the tumor cells in established lung/bone nodules undergo MET. Additionally, future studies are needed to examine the expression of nuclear bFGF/DNA-PK in CTC isolated from blood of TN breast cancer patients.

6. Concluding Remarks and Future Directions

6.1 Summary of findings

The first portion of our studies focused on establishing *in vitro* models of TN breast cancer dormancy/recurrence. Using short term chemotherapy treatment to enrich for drug-resistant tumor cells, we have developed an *in vitro* model of tumor recurrence. In this model, short-term exposure of TN breast tumor cells to clinical-relevant chemotherapy classes/doses enriches for a population of slow-cycling (dormant) tumor cells. Chemotherapy-enriched dormant tumor cells resume proliferation after chemotherapy withdrawal, forming colonies resembling a tumor recurrence.

Next, we identified nuclear bFGF/DNA-PKcs as a key signaling pathway driving TN breast cancer dormancy/recurrence (Fig. 33). We observed upregulation of nuclear bFGF isoforms, but not cytosolic bFGF isoforms, in chemotherapy-enriched dormant TN tumor cells. Nuclear bFGF is necessary and sufficient to drive the survival of dormant tumor cells and establishment of recurrent colonies as confirmed by bFGF knockdown and add back studies. Additionally, we identified DNA-PKcs as a downstream target of nuclear bFGF in chemo-residual dormant TN tumor cells. A small molecule inhibitor of DNA-PKcs reduces both the number of chemo-residual, dormant tumor cells and the number of recurrent colonies in our *in vitro* model of TN breast cancer recurrence. This DNA-PKcs inhibitor likely reduces the number of chemo-residual dormant tumor cells by blocking DNA repair, leading to increased tumor cell apoptosis. Moreover, we also

analyzed gene expression profiles of triple-negative breast cancer patients. Notably, we found that bFGF expression in primary TN breast cancers is associated with tumor recurrence.

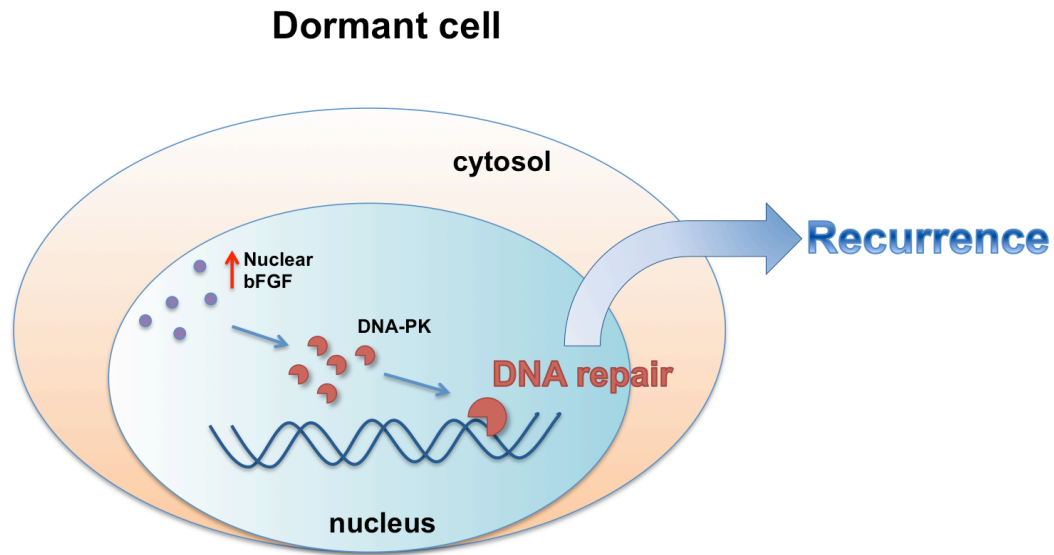


Figure 33: Model of tumor recurrence mechanism. Chemotherapy-enriched dormant TN tumor cells have increased nuclear bFGF expression. Nuclear bFGF upregulates DNA-PK expression and activation. Increased DNA-PK activity accelerates DNA repair. The completion of DNA repair initiates cell regrowth, resulting in tumor recurrence.

Finally, we characterized the properties of recurrent tumor cells evolving after chemotherapy withdrawal in our model. Recurrent cells were more resistant to subsequent chemotherapy challenge than parental tumor cells, and showed broad chemoresistance to different regimens. Such resistance in recurrent tumor cells is likely associated with their increased expression/activation of DNA-PK and elevated DNA repair capacity. We also showed that recurrent tumor cells demonstrate increased

migration, invasion, and EMT, implying an aggressive behavior consistent with recurrent tumor in TN breast cancer patients.

Chemotherapy has been the only systemic treatment of TN breast cancer for a long time. However, our studies suggest that chemotherapy alone may accelerate disease progression by enriching for a chemo-resistant sub-population that can form recurrent tumor with aggressive behavior and expanded therapy resistance. Thus, developing combination therapy targeting both chemo-sensitive and chemo-resistant tumor cells is essential for eradication of TN breast cancer.

6.2 Future studies

To further improve our *in vitro* model of tumor recurrence, we will determine whether other chemotherapies (cisplatin, cyclophosphamide) or sequential therapy combining different regimens also enrich dormant cells that subsequently lead to recurrence. It is important to determine whether dormant and recurrent tumor cells treated with different therapies share common molecular markers. Specifically, gene expression profiles will be obtained from different dormant and recurrent cells and we will seek to identify genes with similar expression changes as potential future therapeutic targets.

After developing a TN breast cancer dormancy/recurrence model and elucidating the underlying mechanism *in vitro*, the next step is to test it *in vivo* using an orthotopic mouse model. Specifically, we plan to inject TN breast cancer cells into the

mammary fat pad of female athymic nude mice. Doxorubicin will be injected IV after the establishment of palpable tumor. When significant shrinkage of tumor is achieved, the residual tumor will be extracted. Dormancy status (p21 expression) and bFGF expression will be examined in residual tumor cells. According to our *in vitro* studies, we expect to see increased level of p21 and bFGF in residual tumor cells. Furthermore, we will confirm the function of nuclear bFGF in tumor dormancy/recurrence in this orthotopic mouse model. Specifically, we plan to inject TN breast cancer cells expressing nuclear bFGF shRNA +/- 23 kDa bFGF vector or control shRNA +/- 23 kDa bFGF vector into the mammary fat pad of female athymic nude mice. Doxorubicin will be injected IV after after the establishment of palpable tumor. The dose of doxorubicin will be carefully determined to reduce tumor volume, but will not eliminate tumor cells[81], providing a window for studying additive effects of doxorubicin + bFGF knockdown. Tumor volume will be measured with a caliper 3x/wk. When complete tumor disappearance is achieved, mice will be followed for tumor recurrence. Time to recurrence will be calculated using Kaplan Meier survival analysis.

Expression of nuclear vs cytosolic bFGF isoforms is determined by alternative translation pathways. Whereas cytosolic bFGF isoforms are regulated by CAP-dependent translation, nuclear bFGF isoforms are regulated by CAP-independent translation. Notably, we observed increased protein levels of nuclear but not cytosolic bFGF isoforms in chemotherapy-enriched dormant tumor cells. Our data suggests that

chemo-residual TN tumor cells may support CAP-independent translation, driving expression of nuclear bFGF/DNA repair. We are currently addressing this important hypothesis. Ultimately, it may be possible to eliminate these chemo-residual dormant tumor cells by targeting the CAP-independent translation pathway.

Based on our demonstration that chemotherapy enriches for bFGF-expressing tumor cells *in vitro*, it will also be important to examine frequency of bFGF protein expression in residual tumor cells from neoadjuvant chemotherapy-treated TN breast cancer patients. Based on our finding that bFGF expression in primary TN breast cancers is associated with tumor recurrence, future studies are needed to determine which bFGF isoforms (nuclear or cytosolic) are associated with tumor recurrence and predicts prognosis in TN breast cancer patients.

To date, it is unclear that whether dormant cells enriched by different chemotherapy agents (doxorubicin or docetaxel) are from the same sub-population, although they have similar properties such as upregulated nuclear bFGF. It is also important to identify unique molecular markers that can be used to isolate chemo-resistant dormant cells from the original population. We plan to perform bFGF staining on untreated TN breast cancer cell lines as well as on untreated patient tumor biopsy samples to address whether bFGF can be used as a molecular marker to identify dormant tumor cells.

Nuclear-localized EGF receptor is a critical determinant of DNA-PK activity[65]. Based on this knowledge, in addition to our current findings, it is intriguing to speculate that nuclear bFGF may control DNA-PKcs expression/activity in a manner dependent on a nuclear bFGF receptor, a topic of current investigation. This possibility is supported by a literature demonstrating that nuclear FGF cooperates with a nuclear FGF receptor to drive gene transcription in neurons[66]. Identifying a bFGF receptor that drives nuclear bFGF/DNA-PK signaling has the potential to define a logical therapeutic strategy for eliminating chemo-residual triple-negative breast cancer cells, thus preventing tumor recurrence.

Previously studies show that nuclear bFGF over-expression drives DNA-PKcs transcription[30]. We will address the hypothesis that nuclear bFGF in chemo-residual TN tumor cells drives DNA-PKcs transcription. Specifically, we will measure DNA-PKcs transcription in control shRNA-expressing cells and bFGF shRNA-expressing cells by luciferase gene reporter assay.

As mentioned above in section 1.4, BRCA mutation is frequently associated with TN breast cancer and increases tumor cell susceptibility to DNA-damaging agents[2]. However, the cell lines used in our studies (SUM159, BT549) have wild type BRCA1[82]. Future studies are needed to examine the effect of blocking DNA-PK activity in BRCA1 mutated TN breast cancer cell lines (SUM149, SUM1315, and HCC1937) because dormant cells in these cell lines are probably more vulnerable to DNA-PK activity

inhibition due to lack of an alternative DNA repair pathway (homologous recombination by BRCA1). Therefore, combination therapy (chemotherapy + DNA-PK inhibitor) may achieve increased efficacy in TN breast cancer patients carrying BRCA1 mutation. Accordingly, BRCA mutation can be a promising molecular marker to justify the use of a DNA-PK inhibitor added to conventional chemotherapy for treating TN breast cancer patients.

Additional studies will pursue understanding the mechanisms of chemoresistance and invasiveness in recurrent tumor cells. Based on our demonstration that nuclear bFGF promotes the survival of dormant cells after chemotherapy and the importance of nuclear bFGF during development of metastasis[25], we will assess the role of nuclear bFGF on chemoresistance and invasiveness in recurrent cells in our model. siRNA targeting nuclear bFGF will be transfected into recurrent cells to achieve transient nuclear bFGF knockdown. Chemotherapy sensitivity will be evaluated by thymidine uptake and clonogenic assay. Invasiveness will be assessed by transwell invasion assay.

In our model, dormant cells and recurrent cells have many different properties (morphology, growth rate, migration, and invasiveness). Mechanisms underlying the conversion from dormant cells to recurrent cells are still unknown. Alterations in pathways regulating cell cycle (Cyclin-dependent kinases) and pathways promoting EMT (Snail, Twist, or TGF β) need to be characterized in the transition of dormant cells to

proliferative recurrent cells. These studies will provide novel approaches to: 1) block dormant tumor cells reentry into cell cycle to prevent local recurrence; and 2) inhibit the transition into “more aggressive” recurrent cells with increased migratory and invasive behavior to prevent distal metastasis in TN breast cancer patients.

6.3 Conclusion

We developed an *in vitro* model of TN breast cancer dormancy/recurrence. Short-term exposure of tumor cells to chemotherapy at clinically relevant doses enriches for a dormant tumor cell population that can regain proliferative ability and establish recurrent colonies, resembling tumor recurrence. Furthermore, we identified a novel signaling axis (nuclear bFGF/DNA-PK) that drives accelerated DNA repair in chemotherapy-enriched dormant TN breast cancer cells. Targeting this axis with either a bFGF shRNA or DNA-PK small molecule inhibitor blocks recurrent colony formation. We found that bFGF expression in tumor samples from TN breast cancer patients predicts five-year tumor recurrence following neoadjuvant chemotherapy treatment. Additionally, we demonstrated that recurrent tumor cells exhibit increased chemotherapy resistance and invasiveness, resembling therapy resistance of recurrent tumors in patients. Collectively, our studies on the mechanisms underlying TN breast cancer dormancy/recurrence provide a novel therapeutic strategy for TN breast cancer treatment.

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Biography

Shenduo Li was born in Shenyang, China on September 21st, 1985 and soon moved to Nanjing, China with his parents Long Li and Ze Wang. He graduated from the High School Affiliated to Nanjing Normal University in 2004. He then attended Peking University major in Clinical Medicine. During this time, he received Elsevier Medical Education Scholarship (2005), Exceptional Medical Student Scholarship (2006, 2008), and Award for Outstanding Academic Performance (2006, 2008). After receiving his medical degree in 2009, Shenduo was recruited to the doctoral training program in Pathology as a Chancellor's Scholar at Duke University in Durham, NC. He pursued his graduate studies under the mentorship of Drs. Robin E. Bachelder and Salvatore V. Pizzo. His doctoral dissertation is entitled "Basic Fibroblast Growth Factor Regulation of Triple-negative Breast Cancer Dormancy/Recurrence". Upon completion of his graduate training, Shenduo plans to work as a postdoctoral fellow in the laboratory of Drs. Robin E. Bachelder and Salvatore V. Pizzo. Ultimately, he plans to pursue a career in academic medicine.

Publications

Li S, Payne S, Wang F, Claus P, Pizzo SV, Bachelder RE. Nuclear basic fibroblast growth factor regulation of triple-negative breast cancer dormancy/recurrence. 2014 (submitted)

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